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~~The~~ role of Phosphoinositide 3-kinase p110δ in mast cell ~~growth factor dependent homeostatic responses and the allergic immune response.~~

A dissertation submitted to the University of London in candidature for the degree of Doctor of Philosophy

By

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Abstract

Class IA phosphoinositide 3-kinases (PI(3)Ks) are critical control elements of signalling cascades with influence over cell survival, proliferation and migration. Mammals have 3 distinct class IA PI(3)K catalytic subunits. One of these, p110δ, is preferentially expressed in leukocytes.

Previous murine genetic studies using strategies which target regulatory elements thereby disrupting class IA PI(3)K signalling indiscriminately of the catalytic subunits have established that PI(3)K are important for Kit signalling and mast cell homeostasis but not for mast cell activation and the allergic response.

We have utilised a mouse model in which p110δ is inactivated by introduction of a germline mutation in the ATP binding site, creating $p110\delta^{D910A}$, mimicking the effects of a pharmacological inhibitor for p110δ.

Bone marrow mast cells (BMMCs) from $p110\delta^{D910A/D910A}$ mice have severe defects in SCF/Kit responses including proliferation, migration and adhesion; additionally BMMCs derived from these mice have a substantial defect in antigen receptor signalling and mast cell degranulation. $p110\delta^{D910A}$ mice have tissue site-selective loss of mast cell populations and a substantial reduction in IgE/Ag-induced passive cutaneous anaphylaxis response. Using p110δ isoform-selective inhibitors it is possible to replicate our genetic strategy and completely block the *in vivo* allergic immune response. Furthermore using such compounds and human mast cells we show that our findings have direct relevance to the human system.

Our data identify p110δ as critical to homeostatic (through its role in growth factor dependent responses) and pathologic phases of mast cell biology (through its role in driving FcεRI-dependent responses) and point towards a promising future for using p110δ inhibitors to treat mast cell-associated pathological disorders.

Statement

This thesis is an account of research conducted at the Ludwig Institute for Cancer Research (University College London Branch), between September 2001 and September 2005. Except where references are given, this thesis contains my own original work, any assistance and contribution of others is highlighted within the text.

Some of the work presented in this thesis has published elsewhere:

Ali, K et al. (2004). "Essential role for the p110delta phosphoinositide 3-kinase in the allergic response." Nature **431** (7011): 1007-11

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It would have been very difficult to complete this thesis if my friends and colleagues had not helped me in so many different ways.

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A comprehensive list of friends and colleagues who have had impact on my PhD and my thesis would extend to several pages, some of these people are unfortunately no longer with us but to everyone I would like to say 'Thank you'.

Finally, I would like to acknowledge the generous support of the MRC and Novartis.

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1.0 Introduction

Abbreviations

Ab	Antibody
BMMC	Bone marrow derived mast cell
BH	Bcr-homology domain
BSA	Bovine Serum Albumin
CTMC	Connective tissue mast cell
cAMP	Cyclic adenosine monophosphate
DAG	Diacylglycerol
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EGTA	ethyleneglycol-bis(2-aminoethyl) N,N,N&',N&',-tetraacetic acid
ERK	Extracellular-signal Regulated Kinase
fMLP	formyl-Met-Leu-Phe
Gab	Grb2-associated binder protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescence protein
GPCR	G-protein coupled receptors
GST	Glutathione-S-Transferase
GTP	Guanine-nucleotide-triphosphate

HTAB	Hexadecyltrimethyl ammonium bromide
IC_{50}	50% inhibitory concentration
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IGF-1	Insulin like growth factor-1
IL-	Interleukin
IP_3	Iositol-1,4,5-triphosphate
IRS-1	Insulin receptor substrate-1
ITAMs	immunoreceptor tyrosine-based activation motifs
JNK	Jun N-terminal kinase
KO	Knock-out
LAT	Linker for activation of T cells
LPS	lipopolysaccharide
LT	Leukotriene
MAPK	Mitogen-Activated-Protein Kinase
MEF	Mouse embryonic fibroblast
MMC	Mucosal mast cell
MC_{TC}	Tryptase, Chymase and carboxypeptidase positive mast cells
MT_T	Tryptase positive mast cell
PAGE	Polyacrylamide Gel Electrophoresis

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PH	Pleckstrin homology domain
PI(3)K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-(4,5)-bisphosphate
PIP_3	Phosphatidylinositol-(3,4,5)-triphosphate
PKA	Protein kinase A
PKB/Akt	Protein kinase-B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PVDF	Polyvinylidene fluoride
pY	Phosphotyrosine
SDS	Sodium Dodecyl Sulfate
SCF	Stem cell factor
SHIP	SH2-domain-containing inositol phosphatase
SH2	Src-homology 2 domain
SLP76	SH2-containing leukocyte protein of 76 kDa
Th	Helper Type T cell

TLC	Thin Layer Chromatography
TLR	Toll-like receptor
TNFβ	Tumor necrosis factor-β
WT	Wild-type

1. Introduction

1.1 The Mast cell and Phosphoinositide 3-kinase

The immune system is a large, complex network of cells, receptors and chemical mediators which maintains the homeostasis of an organism. Scientific discoveries over the last century have helped identify the various components of the immune system and describe how these cells and their mediators protect us against disease.

The protective role of the immune system is tightly regulated and is designed to be self-limiting once the pathologic condition has been resolved, however in the rare instances when this regulation breaks down or in people with genetic predispositions the immune system can promote diseases such as allergy, autoimmunity and even cancer.

The immune system is divided into two broad sections, namely the innate (non-specific) and adaptive (specific). The innate immune response is the frontline against bacterial and viral infections, rapidly responding to foreign material by phagocytosis and by releasing reactive chemical species which non-specifically destroy material within the site of inflammation. In contrast, the adaptive immune response is slower to respond, relying on antigen presenting cells of the innate immune system including macrophages and dendritic cells to help capture and present foreign material which it utilises to mount a highly specific cellular and humoral, T and B cell dependent secondary response upon subsequent exposure.

More recently it has been realised that some cells within the immune system including the mast cell can cross these boundaries between innate and adaptive immunity with roles in both sections of the immune system.

The mast cell is a tissue-resident effector cell of bone-marrow origin that responds to both endogenous and exogenous stimuli with immediate and delayed release of inflammatory mediators. Mast cells have long been implicated in the pathology and mortality of anaphylaxis and other allergic disorders by virtue of their ability to be activated through antigen that binds antigen-specific immunoglobulin-E (IgE) bound to the mast cell surface (Metcalfe, Baram et al. 1997; Wedemeyer and Galli 2000). More recently the mast cell has been linked to pathologies beyond IgE and allergic disease. The developing paradigm for mast cell function integrates critical roles in innate and adaptive immunity together with accessory functions in diverse pathological conditions including autoimmunity and cancer (Lee, Friend et al. 2002; Galli, Kalesnikoff et al. 2005; Littlepage, Egeblad et al. 2005; Nigrovic and Lee 2005; Wedemeyer and Galli 2005).

Phosphoinositide 3-kinases (PI(3)Ks) are a group of enzymes that produce intracellular second messenger lipids and initiate intricate signaling cascades with influence over cell homeostasis and activation (Carpenter and Cantley 1996; Fruman, Meyers et al. 1998; Vanhaesebroeck, Leevers et al. 2001; Vanhaesebroeck, Ali et al. 2005). Recently described gene-targeted mouse models of PI(3)K subunits have highlighted the importance of this group of signal transducers in the immune

system (Okkenhaug and Vanhaesebroeck 2003; Okkenhaug and Vanhaesebroeck 2003; Deane and Fruman 2004; Fruman 2004; Vanhaesebroeck, Ali et al. 2005).

The focus of this work has been to further characterise PI(3)K signaling within mast cells and the allergic cascade. An in depth introduction and historical perspectives on mast cells and PI(3)K are beyond the scope of this section for which the reader is referred to several recent reviews (Carpenter and Cantley 1996; Metcalfe, Baram et al. 1997; Fruman, Meyers et al. 1998; Wedemeyer and Galli 2000; Vanhaesebroeck, Leevers et al. 2001; Puxeddu, Piliponsky et al. 2003{Wymann, 2003 #12; Deane and Fruman 2004; Fruman 2004; Galli, Kalesnikoff et al. 2005; Vanhaesebroeck, Ali et al. 2005).

1.2 Mast cell biology

1.2.1 Origin and development

The mast cell is a 10-20 μm in diameter tissue-resident leukocyte with an *in situ* appearance ranging from ovoid to elongated (Schulman, Kagey-Sobotka et al. 1983; Metcalfe, Baram et al. 1997). Defined by the presence of dense cytoplasmic granules which cause the metachromasia when stained with toluidine blue, mast cells are haematopoietic in origin and differentiate from $CD34^+$ bone marrow progenitor cells (Metcalfe, Baram et al. 1997; Puxeddu, Piliponsky et al. 2003).

Thy-1^{lo} Kit^{hi} $Fc\varepsilon RI^{-ve}$ progenitor cells migrate from the bone marrow into the circulation and under the influence of chemical messengers and cell adhesion

molecules localise into vascularised mucosal and connective tissues, where they undergo final maturation (to become FcεRI^{+ve}) under the influence of stem cell factor (SCF) and the local environment (Metcalfe, Baram et al. 1997; Puxeddu, Piliponsky et al. 2003).

1.2.2 Survival and differentiation

The primary signal which maintains tissue mast cells is SCF which interacts with its receptor Kit (product of the *Kit* proto-oncogene) (Galli, Zsebo et al. 1994{Metcalfe, 1997 #55). Mice lacking functional membrane SCF (Sl/Sl^{d}) or with mutations within the Kit receptor (W/W^{v}) have a severe reduction in tissue mast cells (have less than <1% of wild-type mast cells in the skin and no detectable mast cells in the peritoneal cavity, respiratory tract, gastrointestinal tract, or other sites) (Metcalfe, Baram et al. 1997; Galli, Kalesnikoff et al. 2005). Additionally T-cell derived cytokines such as interleukin (IL) -3, -4, -9 and -10, acting in synergy with SCF, can influence the development of mast cell subsets and the rapid proliferation of these cells under pathological conditions (Metcalfe, Baram et al. 1997; Puxeddu, Piliponsky et al. 2003). In the presence of a normal SCF/Kit system, athymic nude mice lacking a T-lymphocyte system have site-selective loss of intra-epithelial mast cells and are unable to develop jejunal mast cell hyperplasia upon helminthic infection highlighting the vital importance of T-cell cytokines for certain mast cell populations (Austen and Boyce 2001).

1.2.3 Heterogeneity

Mast cells develop *in situ* from circulating hematopoeitic $CD34^+$ progenitor cells which are released from the bone marrow. Studies which have focused on the mouse intestine as a model system for mast cell development have shown that mast cell progenitors migrate from the circulation into the intestinal mucosa a process which is dependent upon the β7-integrin subunit .

Two main rodent mast cell subpopulations, the connective tissue mast cells (CTMC, also known as constitutive mast cells) and the mucosal mast cells (MMC, also known as the reactive mast cells) have been identified (Table 1) and characterised according to morphological and histochemical characteristics (Metcalfe, Baram et al. 1997; Boyce 2003; Boyce 2004; Krishnaswamy, Ajitawi et al. 2006). The CTMC is particularly common in the skin and peritoneal cavity whereas the MMC is most common in the intestinal lamina propria. CTMC and MMC differ in size and histamine content and in function, including responsiveness to secretagogues and drug susceptibility (Metcalfe, Baram et al. 1997). MMC appear to be T-cell-dependent and can rapidly expand upon T-cell-dependent immune responses to certain intestinal parasites. In contrast, CTMC exhibit little or no T-cell dependence and are found in normal numbers in athymic nude mice (Metcalfe, Baram et al. 1997; Austen and Boyce 2001).

Human mast cells also exhibit heterogeneity with differences in size, histochemical properties, quantities of stored mediators, sensitivity to stimulation and drug susceptibility (Table 2) (Metcalfe, Baram et al. 1997). Like in rodents, human mast

cells can be classified into two principle populations according to neutral protease content. Some human mast cells contain measurable levels of tryptase, chymase and carboxypeptidase, these cells are classified as MC_{TC} because they contain both tryptase and chymase (Metcalfe, Baram et al. 1997). In contrast the mast cells called MC_T contain only tryptase (Metcalfe, Baram et al. 1997). The MC_T type mast cell (corresponds most closely to MMC) is predominantly found in the alveolar septa of the lungs and in the small intestinal mucosa, whereas the MC_{TC} is mainly found in the skin and the intestinal sub-mucosa (and corresponds closely to the CTMC) (Metcalfe, Baram et al. 1997).

1.2.4 Activation

Polyvalent antigen cross-linking of immunoglobulin ε (IgE) bound to FcεRI receptors triggers mast cell activation, this results in the release of pre-formed mediators, such as histamine and tryptase, but also in the synthesis and release of newly generated lipid mediators, such as leukotriene $(LT)B_4$, LTC_4 and prostaglandin D_2 and of a large number of cytokines, such as TNFα. Mast cell liberated pro-inflammatory mediators rapidly induce vasodilation and an increase in vascular permeability, brochial mucosa edema, mucus secretion and smooth-muscle contration, they also partcipate in eliciting an inflammatory-cell infiltrate (including neutrophils and eosinophils) which can further exacerbate the immediate hypersensitivity response. (Metcalfe, Baram et al. 1997; Wedemeyer and Galli 2000; Boyce 2003; Puxeddu, Piliponsky et al. 2003; Galli, Kalesnikoff et al. 2005). Mast cells may also be activated by a variety of non-immunological stimuli such as

bacterial proteins and chemical agents including morphine sulphate, substance P, compound 48/80 and the anaphylatoxins C3a and C5a. Responsiveness to the activating stimuli appears to be dependent on the mast cell subset challenged, and the microenvironment (which itself can determine mast cell development) (Metcalfe, Baram et al. 1997).

The exact profile of mediators released is determined by the nature of the activating stimulus (Table 3) and is under the influence of the local environment in which activation takes place (Metcalfe, Baram et al. 1997; Wedemeyer and Galli 2000; Rivera, Cordero et al. 2002; Boyce 2003; Puxeddu, Piliponsky et al. 2003; Galli, Kalesnikoff et al. 2005).

Table 1.1 Mouse mast cells

Characteristics	*Peritoneal Cavity Mast cell*	*Intestinal Mucosa Mast cell*
Alternative names	Connective tissue mast cell	Mucosal mast cell
Size	10-20 μM	5-10 μM
Formaldehyde fixation	Resistant	Sensitive
Staining	Safranin	Alcian blue
T cell dependence in development	No	Yes
Protease content	Chymase (Rat mast cell protease I)	Chymase (Rat mast cell protease II)
Proteoglycan molecular mass	Heparin 750-1000 kDa	Chondroitin sulphate di B 100-150 kDa
Histamine	10-20 pg/cell	1 pg/cell
5-Hydroxytryptamine	1-2 pg/cell	<0.5 pg/cell
Prostaglandin D_2	+	+
Leukotriene C_1	-	++
Activated by		
FcεRI aggregation	Yes	Yes
Compound 48/80	Yes	No
Substance P	Yes	No
Inhibited by sodium cromoglycate	Yes	No

Table adapted from (Metcalfe, Baram et al. 1997)

Table 1.2 Human mast cells

Characteristic	MC_T	MC_{TC}
Neutral protease	Tryptase (10 pg/cell)	Tryptase, Chymase, Carboxypeptidase, Cathepsin G
Granule ultrastructure	Scrolls	Lattice/grating
T-cell dependence	Yes	No
Inhibited by sodium cromoglycate	Yes	No
Distribution %		
Skin	<1	>99
Alveolar tissue	93	7
Nasal mucosa	66	34
Tonsils	40	60
Small intestine		
Mucosa	81	19
Submucosa	23	77

MC_T , Mast cell with tryptase only, MCTC, Mast cell with tryptase and chymase.
Table adapted from (Metcalfe, Baram et al. 1997)

Table 1.3 Major mast cell secreted products

Major mediators stored preformed in cytoplasmic granules	Histamine, serotonin (in murine mast cells), heparin, and/or chondroitin sulfates, neutral proteases (chymases and/or tryptases) major basic protein, many acid hydrolases, cathepsin, carboxypeptidases, peroxidase
Major lipid mediators produced on appropriate activation	PGE_2, PGD_2, LTB_4 and LTC_4, platelet-activating factor
Cytokines released upon appropriate activation	TNF, TGF-β, MIP-1α, VPF/VEGF, FGF-2, LIF, IFN-α, IFN-β, IFN-γ, GM-CSF, MCP-1, IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-9, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25 (and probably many more)
Anti-microbial peptides	Cathelicidin (called LL-37 in human mast cells, and cathelin-related antimicrobial peptide (CRAMP) in murine mast cells)

Table adapted from (Galli, Kalesnikoff et al. 2005)

1.3 Mast cell function in pathology

1.3.1 Allergic disease

1.3.1.1 Th1 and Th2 immune responses

The immune system is a disparate, far-flung collection of individual cells, cell aggregates, immune tissues and organs, thus the regulation of this complex 'organ' system is extremely challenging (Kidd 2003). This complex system is coordinated by T-helper (Th) cells which depending on the nature and the strength of the stimulus differentiate into either Th1 or Th2 subsets and release a large variety of diffusible substances convey messages, give instructions and generally enable the whole system consisting of billions of immune cells to communicate with each other (Kidd 2003).

In the current literature Th1 (cellular, infection fighting) cells and Th2 (humoral, allergy promoting) cells are invoked to rationalize virtually all known patterns of immune response. Th1 cells are hypothesized to lead the attack against intracellular pathogens such as viruses, raise the delayed-type hypersensitivity skin response and fight cancer cells. Th2 cells are believed to emphasize protection against extracellular pathogens (such as parasites). It is also generally accepted that an overreactive Th1 response can generate organ-speific autoimmune disease (e.g. arthritis, multiple sclerosis, type 1 diabeties) (Raz, Eldor et al. 2005). In contrast an imbalance towards a Th2 response is thought to be the underlying cause of allergy and systemic autoimmune disease (Kidd 2003; Agrawal and Bharadwaj 2005). However these stereotypes have proven to be oversimplistic, with the result that the

Th1/Th2 hypothesis is increasingly criticized. Some reasons for these criticisms include the fact that human cytokine activities rarely fall into exclusive Th1 or Th2 patterns, non-regulatory T cells, or the antigen-presenting cells likely can also influence the immune response in a similar manner to Th1/Th2 cells.

Th1 cells secrete a specific cytokine profile including interferon-γ (IFN-γ) and tumor necrosis factor β (TNFβ) both of which are important in helping mount a cellular immune response. Th1 T cell derived cytokines particularly IFN-γ can antagonise the activity of Th2 cells. Interleukin-12 (IL-12), produced by macrophages plays an important role in inducing IFN-γ production (Rogers, Zlotnik et al. 1991; Parronchi, De Carli et al. 1992). In contrast, Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13, which are involved in isotype switching of B cells from immunoglobulin (Ig) M to IgE (IL-4 and IL-13) as well as proliferation and differentiation into antibody-secreting plasma cells (Romagnani, Annunziato et al. 2000). Interleukins-4 and IL-10 are also regulatory cytokines, antagonising the activities of Th1 cytokines (Fig 1.1) (Lester, Hofer et al. 1995).

Overall although the Th1/Th2 hypothesis has its limitations there is evidence for a Th2 cytokine pattern being important in allergic disease. This is evidenced by the success of allergen immunotherapy which has been shown to reduce the production of IL-4 in atopic individuals to levels observed in non-atopic individuals, the skew away from a Th2 pattern is followed by a shift towards a Th1 profile (as concluded

by increased IL-12 mRNA levels in patients receiveing this type of therapy)(Bousquet, Becker et al. 1991).

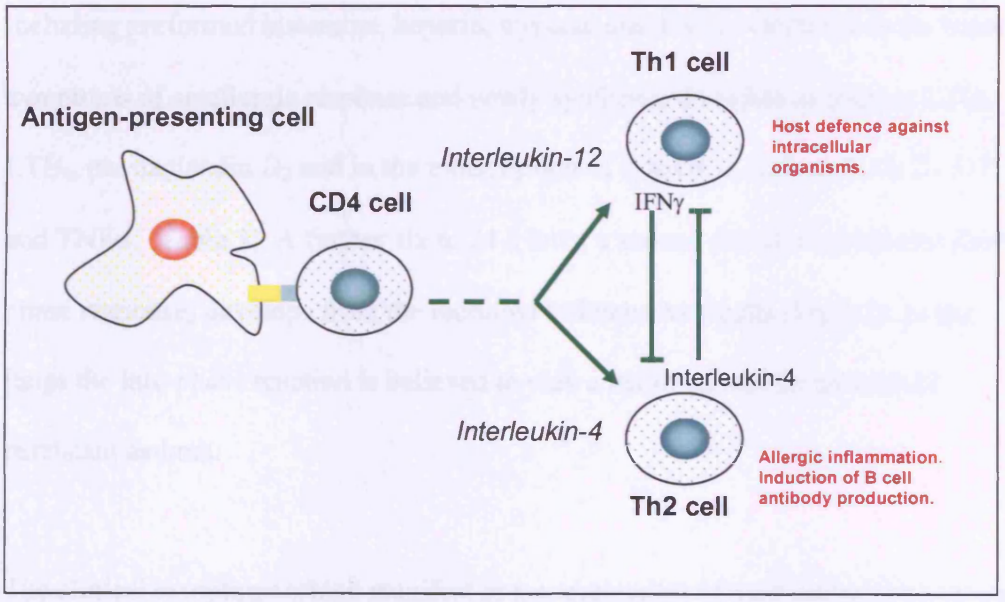


Fig 1.1 Th2 cells predominate in atopic disease. *Differentiation of Th1 and Th2 depends on the cytokines interleukin-12 and interleukin-4, produced by antigen-stimulated precursor CD4 T cells. Th1 and Th2 cells are mutually antagonistic, in atopic individuals the Th2 T cells predominate and promote allergic disease.*

1.3.1.2 Allergic cascade

Initial exposure to allergens leads to sensitization involving B cell isotype switching to IgE production and the formation of plasma cells which release IgE into the circulation (Romagnani, Annunziato et al. 2000). Circulating IgE binds to several

cell types including basophils, eosinophils and the mast cell which has a pivotal role in allergic disease. Binding of IgE to its high affinity receptor expressed on the mast cell surface sensitizes the cell and functions as the mast cell antigen receptor. Subsequent exposure to the antigen leads to cross-linking of the mast cell IgE receptors promoting activation and the release of pro-inflammatory mediators including preformed histamine, heparin, tryptase and TNFα which cause the acute symptoms of an allergic response and newly synthesized mediators such as LTC_4, LTB_4, prostaglandin D_2 and in the transcription of cytokines such as IL-4, IL-5, IL-6 and TNFα. (Table 3). A further six to 24 h later, a second round of symptoms (late-phase response) develops from the recruited inflammatory cells (Fig 1.2), in the lungs the late-phase reaction is believed to play a major role in the genesis of persistant asthma.

The clinical symptoms which manifest as a consequence of mast cell activation are dependent upon site of exposure, the immediate reaction in the skin presents as erythema, edema, and itch; in the lungs as cough, bronchospasm, edema and mucous secretion; and in the gastrointestinal tract as nausea, vomiting, diarrhea and cramping (Wedemeyer and Galli 2000).

1.3.2 Innate and acquired immunity

Mast cells are strategically placed between the internal and external environment and constitute one of the first lines of defence against microbial pathogens (Galli, Maurer et al. 1999). Mast cells express Toll-like receptors (TLR) (Bone marrow-

derived mast cells (BMMCs) express TLR2, TLR4 and TLR6) which are a repertoire of innate immune pattern recognition receptors able to recognise bacterial proteins (Marshall 2004; Marshall and Jawdat 2004). Activation of mast cells through Toll-like receptors leads to release of a specific cytokine/lipid mediator profile responsible for protection against pathogen challenge. The cytokines (including TNFα. leukotrienes and others) released by mast cell activation recruit circulating leucocytes (including neutrophils) with bactericidal properties to the site of infection, (Marshall 2004; Marshall and Jawdat 2004).

Mast cells can also bind parasite-specific IgE, which leads to mast cell activation upon subsequent exposure to parasite antigen or the parasite itself. Activation leads to release of pro-inflammatory mediators as well as cytokines which can augment the adaptive immune response (Marshall 2004; Marshall and Jawdat 2004).

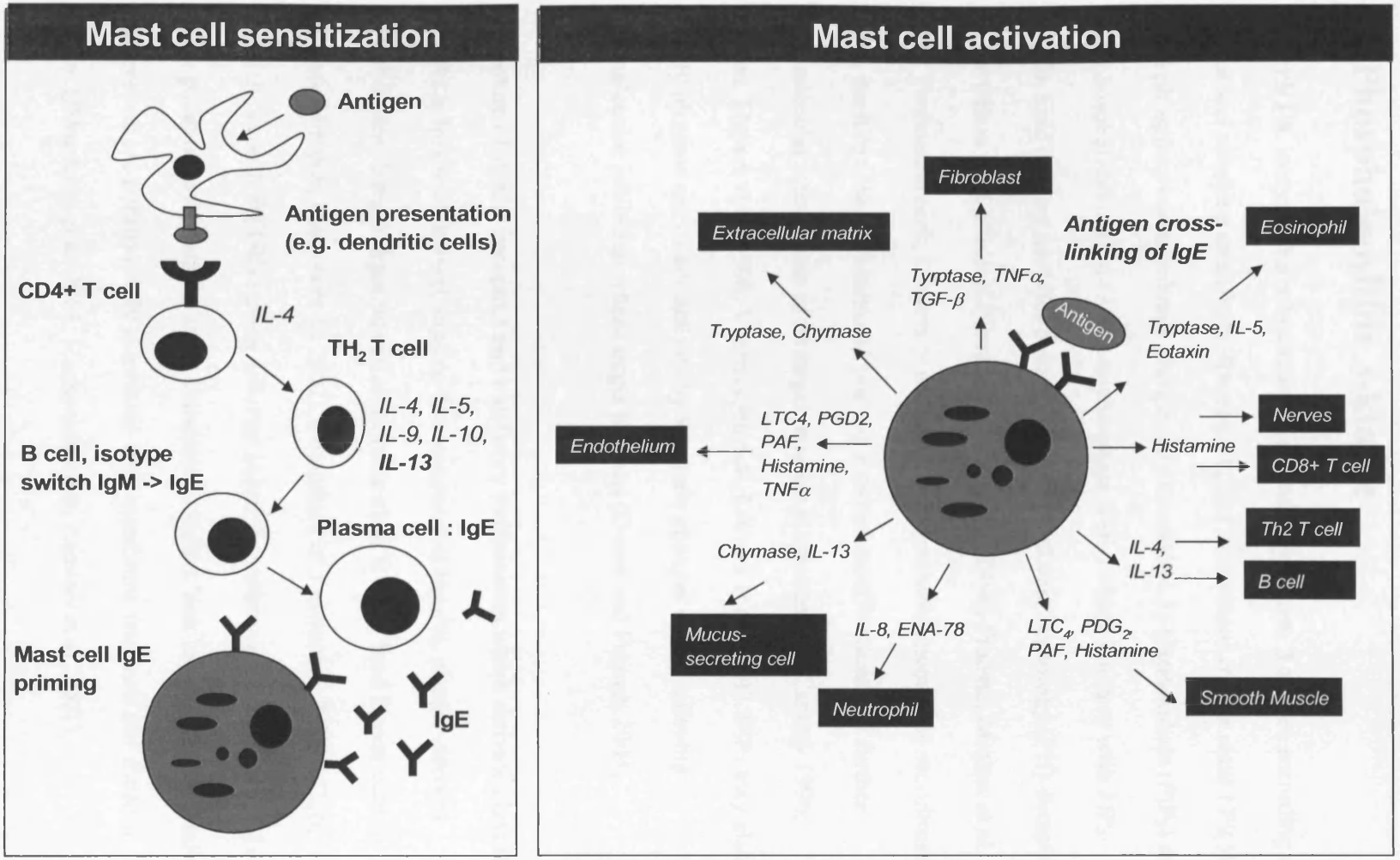


Fig 1.2 The allergic cascade. Mast cell sensitisation occurs following initial exposure to antigens, and is a process in which $CD4^+$ T cells release cytokines (IL-4 and IL-13) which promote B cell maturation and isotype switch from IgM production to IgE. This IgE is captured by IgE receptors on the mast cell surface. Secondary exposure to antigen leads to activation of mast cells and the release of pro-inflammatory mediators which cause the symptoms of allergic disease.

1.4 Phosphoinositide 3-kinase

Eight PI(3)K isozymes have been identified and divided into 3 classes according to adaptor and substrate specificity. Receptor/ligand interactions trigger class I PI(3)Ks which phosphorylate membrane phosphatidylinositol-(4,5)-bisphosphate (PIP_2) to form phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) which interacts with PIP_3-specific lipid binding modules belonging to the pleckstrin homology (PH) domain family within lipid effectors (Carpenter and Cantley 1996; Fruman, Meyers et al. 1998; Vanhaesebroeck, Leevers et al. 2001). PIP_3 promotes membrane recruitment which itself can lead to additional posttranslational modifications and further intermolecular interactions of its target proteins (Carpenter and Cantley 1996; Fruman, Meyers et al. 1998; Vanhaesebroeck, Leevers et al. 2001). PIP_3 may also directly increase enzymatic activity by allosteric changes and/or relieving intramolecular inhibition within target proteins (Deane and Fruman 2004).

The nature of stimuli/receptors and regulatory mechanisms which activate class II PI(3)Ks is less well defined, *in vitro* data suggest that insulin, platelet-derived growth factor, integrin ligation can all activate class II PI3K lipid kinase activity (Vanhaesebroeck, Leevers et al. 2001). Comprised of 3 members, PI(3)K-C2α, -C2β and -C2γ, class II PI(3)Ks have a different substrate preference to class I and III (i.e. utilise phosphatidylinositol > other phosphoinositides), lack known adaptor/binding partners and are constitutively associated with membrane fractions and clathrin (Katso, Okkenhaug et al. 2001; Vanhaesebroeck, Leevers et al. 2001).

Class III PI(3)K is comprised of Vps34p which is thought to be constitutively active, and to maintain a constant level of phosphatidylinositol-3-phosphate lipids. With a specific preference for phosphatidylinositol as a lipid substrate, Vps34p has been implicated in protein sorting to the vacuole/lysozyme and in other membrane transport events, including endocytosis and autophagy (Vanhaesebroeck, Leevers et al. 2001; Wymann and Marone 2005).

The PI(3)K signalling cascade is modulated by phosphoinositide phosphatase activity. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) has 3'-phosphoinositide phosphatase activity and hydrolyses PIP_3 into PIP_2 whereas the SH2-domain-containing inositol phosphatase (SHIP), which has 5'-phosphoinositide phosphatase activity, produces phosphatidylinositol-(3,4)-bisphosphate, which can activate further signalling proteins downstream of PI(3)K (Katso, Okkenhaug et al. 2001; Vanhaesebroeck, Leevers et al. 2001; Wymann, Zvelebil et al. 2003). Work in this thesis focused on the class I PI(3)K family.

1.4.1 Class I PI(3)K

The Class I PI(3)Ks are a family of heterodimeric molecular complexes comprised of a catalytic subunit associated with a regulatory adaptor. Further divided into IA and IB, the class IA PI(3)Ks signal downstream of tyrosine kinase pathways whereas class IB signal downstream of G-protein coupled receptors (GPCRs). The catalytic subunits of all class I members contain a Ras-binding domain and therefore also have the ability to interact with activated guanine-nucleotide-triphosphate (GTP)-

bound Ras (Fruman, Meyers et al. 1998; Katso, Okkenhaug et al. 2001; Vanhaesebroeck, Leevers et al. 2001; Deane and Fruman 2004).

Three class IA ~110 kDa catalytic subunits encoded by distinct genes (*PIK3CA*: p110α, *PIK3CB*: p110β and *PIK3CD*: p110δ) have been identified and couple to one of 5 regulatory/adaptor subunits encoded by 3 genes (*PIK3r1*: p85α, p55α, p50α, *PIK3r2*: p85β and *PIK3r3*: p55γ). The binding of p110 subunit with the regulatory adaptor appears to be constitutive with the adaptor subunit serving a dual purpose both as a mechanism for recruitment and to maintain stability of the p110 subunit holding it in a low activity state (Fruman, Meyers et al. 1998; Katso, Okkenhaug et al. 2001; Vanhaesebroeck, Leevers et al. 2001; Deane and Fruman 2004).

Whereas p110α and p110β appear to have a ubiquitous tissue distribution, the p110δ isoform of class IA PI(3)K has been shown to have a more restricted distribution primarily but not exclusively to the immune system (Chantry, Vojtek et al. 1997; Vanhaesebroeck, Welham et al. 1997; Fruman, Meyers et al. 1998; Katso, Okkenhaug et al. 2001; Vanhaesebroeck, Leevers et al. 2001; Deane and Fruman 2004) (Fig 1.3).

Class IB consists of a single catalytic isoform p110γ (*PIK3CG*) which associates with a p101 (*PIK3r5*) or a newly described adaptor p84 (Suire, Coadwell et al. 2005; Wymann and Marone 2005). Expression of p110γ is restricted mainly to the

haematopoietic system, with lower levels expressed within smooth muscle, endothelial cells and cardiomyocytes (Wymann and Marone 2005).

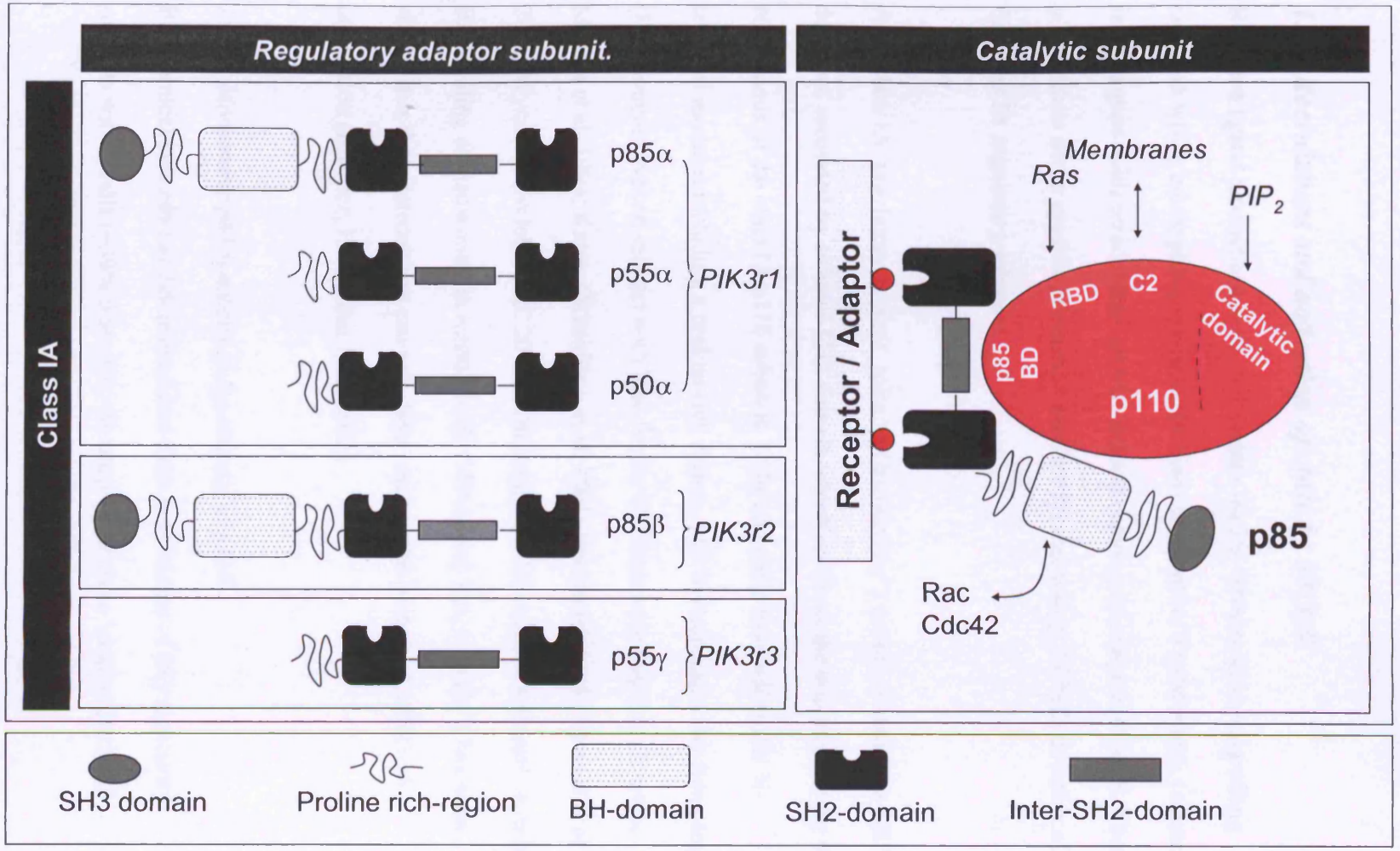


Fig 1.3 Class IA PI(3)Ks. Class IA PI(3)K is composed of five distinct regulatory subunits which couple to one of three distinct catalytic subunits. Class IA PI(3)Ks are recruited to phosphotyrosine residues within the sequence YxxM on phosphorylated receptors and adaptor proteins.

1.4.2 Recruitment and activation of class IA PI(3)K

Receptor/ligand interactions at the cell surface lead to tyrosine kinase signalling cascades which create phosphorylated tyrosines (pY) within YxxM motifs (where x is any amino acid) on activated growth factor receptors and adaptor proteins. These pY residues act as specific interaction sites for Src-homology 2 (SH2) domains of the class IA regulatory subunits.

All 5 class IA regulatory subunit isoforms (encoded by 3 genes) contain two SH2 domains, separated by an inter-SH2 domain which serves as the docking site for the N-terminus of the class IA p110 subunits. p85α and p85β have additional N-terminal modules including a proline-rich region, SH3 domain and a Bcr-homology (BH) domain and can interact with Rho family GTPases including Rac (Fruman, Meyers et al. 1998; Katso, Okkenhaug et al. 2001; Vanhaesebroeck, Leevers et al. 2001; Wymann, Zvelebil et al. 2003). Although class IA catalytic subunits contain Ras binding domains and can interact with GTP-bound Ras, recently it has been shown that these interactions can only take place in the context of a SH2-pY interaction (Jimenez, Hernandez et al. 2002).

1.4.2.1 Monomeric p85 functions independently of p110

Biochemical analysis has determined that there is an excess of p85 regulatory subunits within cells (~30% free p85), the purpose of these 'monomeric' p85

subunits has not been fully determined (Sung, Sanchez-Margalet et al. 1994; Ueki, Fruman et al. 2002).

Part of the biological functions of 'free' p85 may be to drive lipid kinase independent functions. Evidence for this has been shown in T cells where it was found that expression of a membrane-localised p85 lacking the ability to associate with p110 (Δp85) potently upregulated interleukin-2 production in Jurkat and normal peripheral T cells. This feature of p85 was found to be Rac-dependent and is not inhibited by wortmannin (Kang, Schneider et al. 2002).

Monomeric p85 may also attenuate PI(3)K activity by competing with p85/p110 for pY residues (Sung, Sanchez-Margalet et al. 1994; Ueki, Fruman et al. 2002). Recently using a green fluorescence protein (GFP)-tagged p85α (EGFP-p85α) in the context of insulin like growth factor-1 (IGF-1) signalling, monomeric p85α has been shown to be involved in forming a sequestration complex with insulin receptor substrate-1 (IRS-1) and thereby acts as a mechanism for limiting IRS-1/PI(3)K signalling (Luo, Field et al. 2005). After 5-10 min of IGF-1 stimulation, monomeric p85 and IRS-1 assemble preferentially into large complexes in the cytosol which do not produce PIP_3. These interactions appear to depend on the NH_2-terminal SH3 and BH domains of p85α. Indeed, p55α and p50α which lack these domains did not share this function with p85α (Luo, Field et al. 2005). Furthermore the sequestration mechanism for limiting PI(3)K activity also appears to be stimulus-specific and was

not observed upon platelet derived growth factor (PDGF) stimulation of cells (Luo, Field et al. 2005).

1.4.2.2 GPCR modulation of PI(3)K activity

A further mechanism for modulating the PI(3)K pathway as been described and relates to GPCR signalling. Biochemical evidence has shown that the p110β isoform of class IA PI(3)K can interact with the βγ subunits of $G_{i/o}$ family GPCRs. *In vitro*, lipid kinase activity of p110β can be synergistically increased by a pY peptide in combination with Gβγ. To date this feature of class IA PI(3)K appears to be exclusive to p110β as experimental data suggests that p110α and p110δ appear insensitive to this type of stimulation (Katada, Kurosu et al. 1999; Maier, Babich et al. 1999). In contrast to activating class IA PI(3)K, GPCR ($G\alpha_q$ subunit) signalling has also been shown to selectively antagonise p110α lipid kinase activity with no effect on p110β kinase activity (Ballou, Lin et al. 2003). The physiological role of the interactions of class IA with GPCRs remains unclear but may have important implications for diverse immunological responses including leukocyte chemotaxis and activation (Sadhu, Dick et al. 2003; Puri, Doggett et al. 2004; Condliffe, Davidson et al. 2005; Puri, Doggett et al. 2005; Thomas, Smith et al. 2005).

1.4.3 Recruitment and activation of class IB PI(3)K

Ligand-triggered activation leads to translocation of p110γ to activated GPCRs where it is stimulated by its interaction with βγ subunits (Stoyanov, Volinia et al.

1995; Stephens, Eguinoa et al. 1997). Work to identify the relationship between the p101 adaptor protein and the p110γ subunit has revealed that unlike class IA members for which the p85 regulatory subunit is essential for pY interactions, p110γ can be directly stimulated by activated Gβγ (Maier, Babich et al. 1999). The purpose of the p101 subunit appears to be important for lipid substrate specificity (Maier, Babich et al. 1999). Whereas p110γ can interact with and be activated by $G_{\beta\gamma}$ subunits directly, under these conditions p110γ converts phosphatidylinositol primarily into phosphatidylinositol-3-phosphate and very small amounts of phosphatidylinositol-(3,4,5)-triphosphate, however within a heterodimeric complex with p101, p110γ primarily catalyses the formation of phosphatidylinositol-(3,4,5)-triphosphate (Maier, Babich et al. 1999).

Similar to class IA PI(3)Ks, p110γ also contains a Ras binding domain, however although some progress has been made in understanding how class IA interact with this important oncogene the mechanism and importance of p110γ interactions with Ras remain as yet not well-defined.

1.4.4 PI(3)K adaptor proteins

PI(3)K activity is recruited to a large array of activated receptors, many of these are tyrosine kinase receptors able to directly interact with the SH2 domains of the class IA regulatory subunits (Kit, PDGF-receptor etc). However there are an equally large number of receptors which are reliant on PI(3)K activity but unable to directly interact with these SH2 domains (including for example the B-cell antigen receptor, FcεRI, interleukin-3 receptor etc). These receptors rely on recruiting adaptor

proteins which serve as scaffolds that link PI(3)K activity to activated receptors (Wymann, Zvelebil et al. 2003).

Adaptor protein interactions with class IA PI(3)K are reliant on YxxM motifs which are phosphorylated either directly (via intrinsic receptor tyrosine kinase activity) or indirectly via non-receptor tyrosine kinases (e.g. Jak, Syk, Fyn). The insulin receptor substrate (IRS 1-4) and Grb2-associated binder (Gab 1-3) adaptor proteins are typical of this type of adaptor (Wymann, Zvelebil et al. 2003). Both contain multiple YxxM motifs and PIP_3-interacting PH domains which can facilitate membrane translocation (Razzini, Ingrosso et al. 2000; Rodrigues, Falasca et al. 2000). Loss of Gab or IRS adaptor proteins through genetic modification leads to attenuation of receptor (and pY)-associated PI(3)K activity (Myers, Backer et al. 1992; Gu, Saito et al. 2001).

1.5 Gene-targeting of PI(3)K subunits

Gene targeting has been used to as a tool for dissecting molecular function from *in vitro* cell-based systems through to whole organisms. Recently several gene-targeted PI(3)K genetic models been described that have helped to dissect the PI(3)K signalling pathway within the immune system. This section provides a summary of genetic models and brief details relating to published immunological phenotypes in cells other than mast cells (the latter will be discussed separately).

1.5.1 Regulatory subunit knock-out (KO) mice ('p85 KO mice')

Of the five class IA regulatory/adaptor proteins all but one (*PIK3r3*, encoding p55γ) have been subjected to gene targeting and are commonly referred to as 'p85 KO mice'.

1.5.1.1 Complexities of targeting regulatory subunits

The p85 regulatory subunits are essential for maintaining stability of the PI(3)K complex (Yu, Zhang et al. 1998). Genetic disruption of genes which encode the regulatory subunits leads to a concomitant decrease in p110 expression proportional to the reduction in the relative level of regulatory adaptor(s) (Vanhaesebroeck, Ali et al. 2005). Although p85 KO mice have a number of immunological phenotypes thought to be a consequence of this reduction in PI(3)-*kinase* activity, the interpretation of these phenotypes is complicated by evidence of deregulation within the class IA compartment. Apart from disrupting p110 expression it appears that genetic manipulation of the regulatory adaptor compartment can also alter the expression of the remaining adaptor subunits leading to upregulation of the non-targeted adaptor proteins (Vanhaesebroeck, Ali et al. 2005).

The p85 regulatory subunits may also influence the expression, stability and recruitment of other important proteins interacting with the PI(3)K pathway, including the phosphatases PTEN and SHIP. Previous work in mouse embryonic fibroblasts (MEFs) has shown that PTEN protein expression is sensitive to p85 levels. Heterozygous p85α null mouse embryonic fibroblasts (MEFs) been reported

to have a two-fold increase in PTEN expression which was not observed in homozygous p85α null cells (Ueki, Fruman et al. 2002). It is possible that this deregulation of PTEN expression is related to the *in vitro* transformation of the embryonic fibroblasts by introduced oncogenes.

SHIP is known to associate with p85 upon FcγRII activation which can co-aggregate with the B cell receptor and the mast cell antigen receptor upon antigen crosslinking (Gupta, Scharenberg et al. 1999). The impact of regulatory subunit deletions on the SHIP or PTEN phosphatases in immunological cells has not been assessed to date.

Overall the data relating to immunological phenotypes of the various p85 KO mice (outlined below) strongly suggest that class IA PI(3)K has both positive and negative roles in immunological function.

1.5.1.2 p85α is essential for B cell development and function

Disruption of p85α, which appears to be the most abundant regulatory subunit, leads to impaired B cell development and function (Fruman, Snapper et al. 1999; Suzuki, Terauchi et al. 1999; Suzuki, Matsuda et al. 2003; Hess, Donahue et al. 2004). B cells isolated from p85α KO mice have a severe attenuation in antigen receptor-stimulated proliferation and an increased propensity towards apoptosis. Proliferation in response to the polyclonal B cell mitogens lipopolysaccharide (LPS) and anti-CD40 is also impaired but to a lesser extent.

In vivo p85α deletion leads to partial block in early B cell development at the pro-B to pre-B transition, a marked reduction in the number of mature splenic B cells, and a nearly complete absence of the B1 subset of mature B cells. These mice also fail to mount an effective antibody response to T-independent type II antigens.

Disruption of the *PIK3r1* appears to have no impact on T cell development and proliferation (Fruman, Snapper et al. 1999; Suzuki, Terauchi et al. 1999).

1.5.1.3 Class IA PI(3)K maintains Th1/Th2 balance through dendritic cell cytokine production

Class IA PI(3)Ks also appear to have an important role in maintaining the Th1/Th2 balance through its negative role in dendritic cell (DC) function. *In vitro* LPS-stimulated DCs isolated from p85α KO mice have a modest reduction in PI(3)K activity (as assessed by protein kinase B (PKB) phosphorylation) with an associated increase in IL-12 production. Constitutive overproduction of IL-12 (the nature of the *in vivo* stimulus has not been identified) leads to an *in vivo* Th1 skew. The consequence of this altered Th1/Th2 balance include increased resistance to *Leishmania major* infection (which is dependent upon a Th1 response) but increased susceptibility to infections that require a host Th2 response (such as *Strongyloides venezuelensis* parasite infection) (Fukao, Tanabe et al. 2002; Fukao, Yamada et al. 2002).

1.5.1.4 p85β is a negative regulator of T cell function

p85β KO mice do not appear to have immunological defects severe as those reported in the p85α KO. This may in part be a result of compensation by the more dominant p85α isoform, however analysis of T cells from the p85β KO suggests that there maybe qualitative as well as quantitative differences between p85α and p85β (Deane, Trifilo et al. 2004). T cells lacking p85β are hyper-responsive to anti-CD3 stimulated proliferation and survival, even though PI(3)K activity (as assessed by PKB phosphorylation) appears unaffected (Vanhaesebroeck, Ali et al. 2005). The reason for this difference is unclear but maybe a consequence of the loss of as yet unknown p85β-specific protein-protein interactions which play a negative role in T cell responses lost upon of deletion of p85β (Deane, Trifilo et al. 2004).

1.5.2 Catalytic subunit, 'p110' KO mice

All class I PI(3)K p110 subunits have been subjected to gene targeting. Disruption of p110α or p110β is embryonic lethal and has precluded analysis of these isoforms within the immune system (Bi, Okabe et al. 1999; Laffargue, Calvez et al. 2002). The remaining class I isozymes p110δ and p110γ have been targeted using different genetic strategies and have all produced viable mice with various immunological defects (Hirsch, Katanaev et al. 2000; Li, Jiang et al. 2000; Sasaki, Irie-Sasaki et al. 2000; Clayton, Bardi et al. 2002; Jou, Carpino et al. 2002; Okkenhaug, Bilancio et al. 2002; Patrucco, Notte et al. 2004).

1.5.2.1 p110δ is critical to the adaptive immune response

B cells in which p110δ has been genetically inactivated or deleted have defects in development, activation and antibody response similar to those reported in p85α KO mice (Clayton, Bardi et al. 2002; Jou, Carpino et al. 2002; Okkenhaug and Vanhaesebroeck 2003). p110δ also appears to have a role downstream of the T cell antigen receptor (TcR). Purified $CD4^+$ T cells isolated from $p110\delta^{D910A/D910A}$ mice expressing a catalytically inactive form of p110δ have a reduced anti-CD3-stimulated proliferation. CD28 costimulation provides an important second signal in TcR-regulated cytokine production, proliferation and survival. The intracellular domain of CD28 contains a specific tyrosine residue (Y710) that allows CD28 to directly couple to Class IA PI(3)K. Mutation of CD28 such that it can no longer interact with class IA PI(3)K (Y170F) specifically disrupts CD28-mediated survival signals without affecting CD28-dependent proliferation or IL-2 secretion (Okkenhaug, Wu et al. 2001). Consistent with these results, T cell defects in IL-2 production and anti-CD3-stimulated proliferation in $p110\delta^{D910A/D910A}$ mice can be overcome, or even enhanced by CD28 costimulation (Okkenhaug, Bilancio et al. 2002).

1.5.2.2 p110δ is important for neutrophil responses

p110δ is part of the class IA family of PI(3)K which are couple via regulatory adaptor subunits to specific phosphotyrosine sequences (within the consensus YxxM) downstream of tyrosine kinase signaling; therefore in theory p110δ does not

have the capacity to directly interact with GPCR signaling, this is in contrast to p110γ which contains a Gβγ interaction domain. However there are reports that p110δ may also play a role in neutrophil trafficking and activation stimulated by chemokine molecules such as formyl-Met-Leu-Phe (fMLP) which activates GPCRs. Pharmacological blockade or genetic deletion of p110δ leads to attenuated fMLP-stimulated neutrophil activation, LT (leukotriene)B_4 -induced migration and neutrophil accumulation in a model of LPS-induced acute lung injury (Puri, Doggett et al. 2004). p110δ appears to participate not only in leukocyte migration to chemo-attractants but also within endothelial cells where it is important in tumour necrosis factor-α (TNFα)-stimulated E-selectin-dependent adhesion of neutrophils to the vascular endothelium (Puri, Doggett et al. 2005).

It is important to mention that that the role of p110δ within neutrophil activation and chemokine/GPCR-directed migration is contentious, amongst other because there is no biochemical evidence to suggest that p110δ couples directly to chemokine or GPCR signalling. It is possible though that p110δ indirectly couples to GPCR-induced signalling and biological responses (see also paragraph 1.5.3.3).

1.5.2.3 p110γ is critical for driving PI(3)K-dependent GPCR chemotaxis and oxidative burst

Inflammation involves the activation of leukocytes and the release of a host of pro-inflammatory mediators including chemokines which recruit other immune effectors and thereby exacerbate the immune response.

Leukocytes sense and migrate directionally following gradients of chemo-attractant (many of which signal through GPCRs) towards sites of inflammation. The sense of direction is pivotal for cell migration, events at the leading edge of a cell are clearly distinct from those at the trailing edge; subcellular localisation is essential for defining morphological polarity during chemotaxis (Procko and McColl 2005). The process of chemotaxis requires an ordered projection of a pseudopod from the cells leading edge, with the involvement of massive cytoskeletal reorganisation and co-ordinated integrin attachment and detachment at the leading and trailing edges of a cell. Although the precise details remain unclear the involvement of PI(3)K activity in the process of chemotaxis is overwhelming. In cells stimulated with chemoattractants the net PI(3)K (PIP_3 gradient) activity is confined to the leading edge of the cell. Chemokine receptors signall through GPCRs and therefore it is expected that PI(3)K activity downstream of these receptors would most likely be provided by the p110γ isoform of PI(3)K (Stephens, Ellson et al. 2002).

p110γ couples specifically to Gαi type GPCR receptors and has been identified as critical for leukocyte pathfinding. Chemokine activation of p110γ generates a PIP_3

gradient which polarizes a cell towards the chemokine stimulus; this PIP_3 gradient has been identified to be of crucial importance in directing the migration of leukocytes towards chemoattractants. The chemotactic migration of isolated neutrophils, macrophages and eosinophils from p110γ null mice is substantially (but not completely) impaired (Hirsch, Katanaev et al. 2000; Sasaki, Irie-Sasaki et al. 2000; Pinho, Souza et al. 2005; Thomas, Smith et al. 2005).

Apart from its critical role in immune cell chemotaxis, p110γ has an important role in GPCR-stimulated leukocyte respiratory burst in neutrophils and mast cells (discussed later, see section 1.8.1) (Hirsch, Katanaev et al. 2000; Laffargue, Calvez et al. 2002; Condliffe, Davidson et al. 2005). The defect in mast cell activation is thought to be responsible for resistance to systemic anaphylaxis challenge directed through the FcεRI receptor. p110γ also appears to be important for T cell proliferation and survival both of which are reduced in p110γ null mice (Sasaki, Irie-Sasaki et al. 2000).

p110γ null mice have been found to be less responsive in several disease models including LPS-induced acute injury (accumulate substantially reduced numbers of leucocytes), allergic pleurisy (impaired eosinophil accumulation and survival), delayed type sensitivity (defects in dendritic cell homing to peripheral lymph nodes) and rheumatoid arthritis (defective neutrophil migration). In most if not all of these disease models a defect in leukocyte directional migration (which leads to severe defects in leukocyte homing and infiltration) is central to the reduced responsiveness

of p110γ null mice (Hirsch, Katanaev et al. 2000; Sasaki, Irie-Sasaki et al. 2000; Del Prete, Vermi et al. 2004; Camps, Ruckle et al. 2005; Pinho, Souza et al. 2005).

Recently it has been shown that p110γ expressed within the endothelium has an important role in assisting the trafficking of leukocytes to sites of inflammation. p110γ null mice reconstituted with wild-type (WT) neutrophils retain a 45% reduction in neutrophil accumulation following acute lung injury. WT neutrophils have a 70% reduction in attachment and a 17-fold increase in rolling velocities on p110γ null microvessels in response to TNFα. The defect is thought to relate to a deficiency in selectin-mediated adhesion and no doubt contributes towards the severity of the defects in neutrophil accumulation reported in p110γ null mice following acute lung injury and most likely contributes to the reduced responsiveness of p110γ null mice in the disease models mentioned above (Puri, Doggett et al. 2005).

1.5.3 PI(3)K isoform-specific functions

1.5.3.1 Regulatory isoforms have distinct functions

Class IA regulatory isoforms are the product of three distinct genes which encode five different adaptor proteins (Vanhaesebroeck, Leevers et al. 2001). Regulatory adaptors all have a common p110 binding site but have distinct N-terminal regions which allow for additional protein-protein interactions which may differ between the p85 isoforms (Vanhaesebroeck, Leevers et al. 2001).

Deletion of p85α, most likely the most abundant regulatory isoform, leads to attenuated B cell proliferation and survival but appears to leave T cell responses intact (Fruman, Snapper et al. 1999; Suzuki, Terauchi et al. 1999; Suzuki, Matsuda et al. 2003; Hess, Donahue et al. 2004). In contrast p85β appears non-essential for B cell function but is necessary for modulating T cell responses. Deletion of p85β enhances T cell proliferation and survival (Deane, Trifilo et al. 2004).

Class IA regulatory subunits have no kinase activity and there is no data to suggest that they specifically couple to distinct p110 subunits, therefore p85 specific functions uncovered in these genetic studies are most likely the result of differential protein-protein interactions between the various regulatory subunits
B and T cell antigen receptors, unlike tyrosine kinase receptors, have no direct PI(3)K binding sites and PI(3)K activity is recruited to these receptors through adaptors proteins. It is known that B and T cell receptors recruit different protein tyrosine kinases which phosphorylate distinct adaptor proteins upon activation.
It is not known is whether these adaptor proteins associate with different p85 species. This is an area which requires further investigation.

1.5.3.2 p110δ-specific functions

B cells are extremely senstivive to deletion or inactivation of the p110δ subunit. Several reasons including quantitative and qualitative differences between the p110 subunits may account for this sensitivity, p110δ may be the 'dominant' class IA PI(3)K expressed in B cells, as a consequence this may mean that PI(3)K dependent

receptors such as the BCR, in the absence of p110δ may not have access to sufficient PI(3)K to reach a PIP_3 threshold in order to drive a functional response (Chantry, Vojtek et al. 1997; Vanhaesebroeck, Welham et al. 1997) (Clayton, Bardi et al. 2002; Jou, Carpino et al. 2002; Okkenhaug, Bilancio et al. 2002). Qualitative differences between p110 subunits may also be of importance including differences in activation by Ras or intrinsic differences in protein kinase activity(Vanhaesebroeck, Higashi et al. 1999).

1.5.3.3 Cooperation between Class I PI(3)K in neutrophil activation and migration

Genetic data indicate that although p110δ and p110γ have distinct roles in leukocyte biology which is primarily a consequence of the different pathways (tyrosine kinase and GPCR signalling) to which these distinct PI(3)K isoforms couple, there is evidence to suggest that p110δ and p110γ may have overlapping roles in certain leukocyte responses such as neutrophil activation and chemotaxis (Puri, Doggett et al. 2004; Puri, Doggett et al. 2005).

Chemokines activate GPCR receptors and therefore any PI(3)K-dependent functions through such receptors are thought to be translated solely through the p110γ isoform (Wymann, Bjorklof et al. 2003). p110γ null neutrophils have an attenuated chemotaxis which leads to substantially reduced accumulation of these cells upon acute lung injury in p110γ null mice. Part of the mechanism behind these neutrophil

defects appears to be impaired Rac activation and F-actin accumulation at the leading edge.

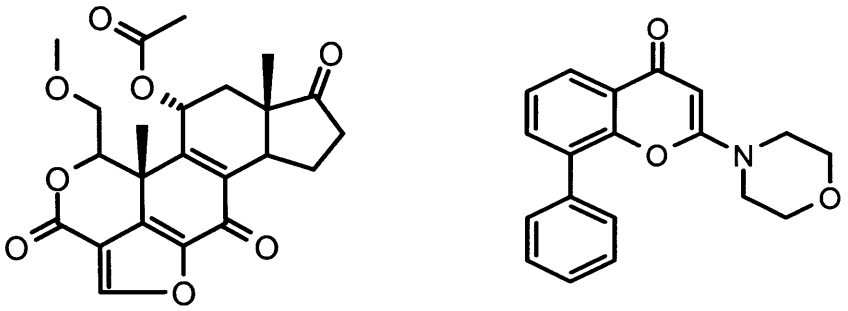
Analysis of p110δ-deficient neutrophils indicates that this GPCR-insensitive PI(3)K isoform may also in some way have a role chemokine responses thought to be p110γ-dependent. Chemokine-stimulated p110δ null neutrophils have impaired chemotaxis and activation (Sadhu, Dick et al. 2003); however unlike in p110γ null cells, F-actin synthesis was not blocked in these cells suggesting that p110δ may affect the same functional responses but in a different way to p110γ (Sadhu, Dick et al. 2003).

Recently published data provides a plausible hypothesis to explain how both of these distinct PI(3)Ks can influence the same phenomenon by working differently within the same pathway. It is now thought that GPCR stimulation can activate both class IB p110γ and class IA PI(3)K isoforms sequentially in a two-step process. Initial activation of GPCR receptors is thought to activate a burst of p110γ-dependent PI(3)K activity which recruits the PH domain containing guanine-nucleotide exchange factor p-Rex1. Recruitment of pRex1 is thought to activate Rac which can then facilitate the activation of p85-bound class IA PI(3)K isoforms which can then further augment PI(3)K activity. There is also evidence for induction of global tyrosine phosphorylation upon GPCR stimulation, which might then lead, in one way or another, to activation of class IA PI3K activity (Condliffe, Davidson et al.

2005). Thus neutrophil chemotaxis and activation may require both class IA and IB working together in distinct ways.

1.6 Pharmacological interference with PI(3)K function

The availability of pharmacological tools has greatly facilitated dissection of the PI(3)K signalling pathway. Wortmannin and LY294002, two first-generation structurally distinct broad spectrum PI(3)K inhibitors have been used widely for this purpose (Fig 4). Both compounds target the ATP binding pocket within the kinase domain and have little selectivity for distinct PI(3)K isoforms (Knight, Chiang et al. 2004). Wortmannin is more potent than LY294002 and has an IC_{50} value for PI(3)K within the low nanomolar range and interacts in a covalent manner with the catalytic site (alkylates a Lys within the ATP-binding site). LY294002 in contrast is an ATP-competitive inhibitor with potency within the low micromolar range. Both compounds have low specificity at higher concentrations and are able to inhibit a number of other protein kinases (Arcaro and Wymann 1993; Thelen, Wymann et al. 1994; Ward, June et al. 1996; Wymann, Bulgarelli-Leva et al. 1996; Stein and Waterfield 2000; Knight, Chiang et al. 2004).



Wortmannin *LY294002*

Fig 4. The broad spectrum PI(3)K inhibitors wortmannin and LY294002

Second generation PI(3)K inhibitors have increased specificity and potency and include a range of compounds which have selectivity for one or more of the class I PI(3)K family and are described in a recent review (Prestwich 2004). Recently a pharmacological inhibitor (IC87114) which selectively targets the p110δ PI(3)K has been described (world patent no. WO0181346) (Fig 5). IC87114 has been described as an ATP-competitive inhibitor able to selectively inhibit p110δ activity. The IC_{50} of IC87114 for p110δ inhibition is 0.5 μM whereas the IC_{50} values for p110α, p110β and p110γ are reported to be >100, 75, and 29 μM, respectively. Thus IC87114 is 58-fold more selective for p110δ over p110γ, and over 100-fold selective relative to p110α and p110β (Sadhu, Masinovsky et al. 2003).

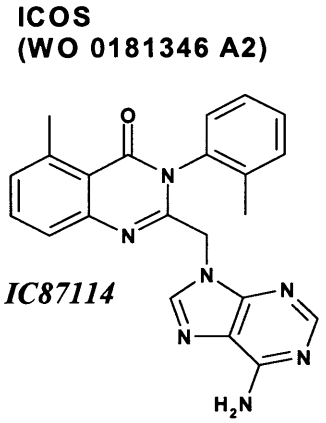


Fig 5. The p110δ-selective inhibitor IC87114

1.7 Role of PI(3)K in mast cell homeostasis

Various approaches have been used to define the role of PI(3)K in mast cell homeostasis including pharmacological inhibitors, expression of dominant-negatively acting proteins and gene targeting. Table 4 lists some phenotypes reported from the various gene targeted PI(3)K mutant mice within a mast cell context.

1.7.1 The Kit receptor is essential for mast cell homeostasis

The receptor tyrosine kinase Kit is critical to mast cell homeostasis. Loss of Kit expression or reduction in Kit kinase activity leads to severe disruption of tissue mast cell numbers. Kit is activated by receptor crosslinking by its cognate ligand

SCF. Activation is followed by receptor auto-phosphorylation on tyrosine residues providing docking sites for specific SH2 domain-containing proteins which once active promote cell proliferation and survival (Metcalfe, Baram et al. 1997).

The Kit receptor is capable of direct and indirect interactions with PI(3)K. Two sites which facilitate Kit interaction with PI(3)K are the juxtamembrane Src binding sites (Y567 and Y569) and the more distal direct PI(3)K binding site Y719 (Fig 1.6). Other interactions of Kit with PI(3)K are possible through adaptor proteins such as Grb2-associated binder protein (Gab2) (Ronnstrand 2004).

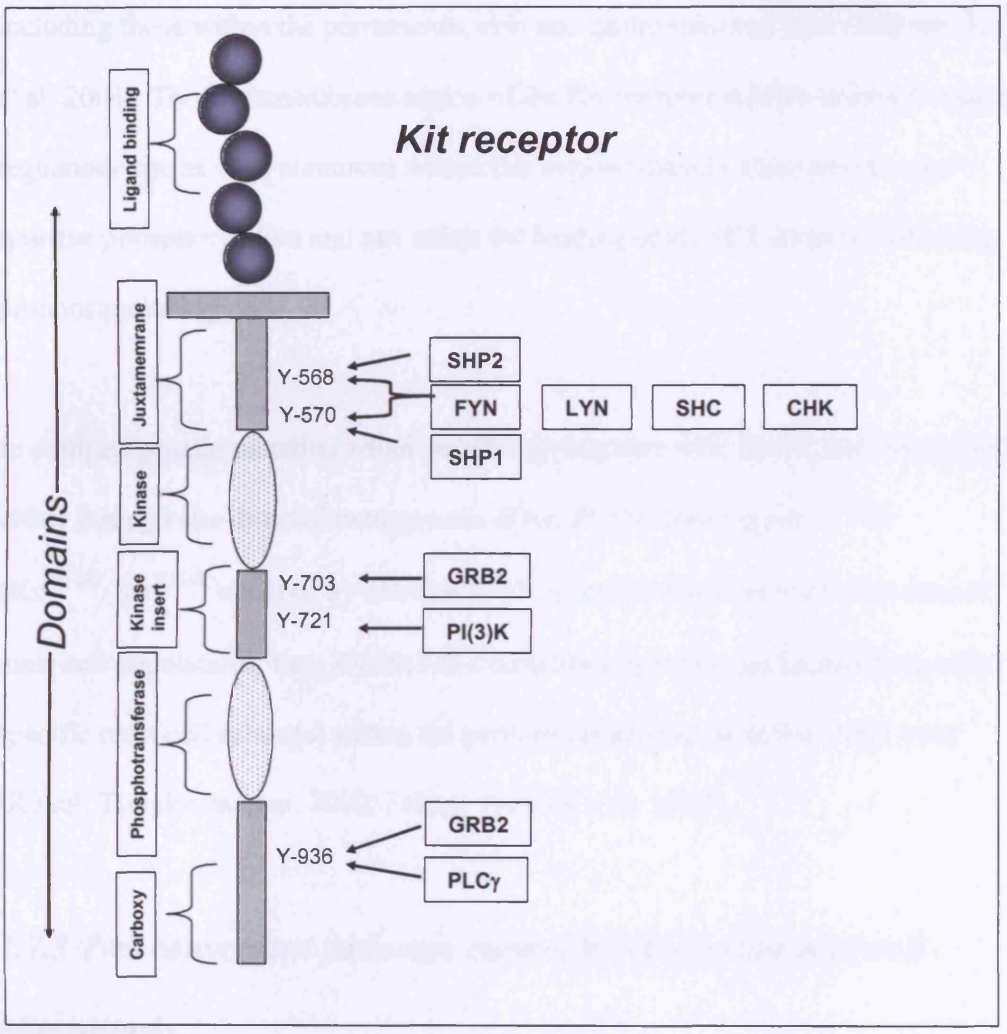


Fig 1.6 The (human) Kit receptor.

1.7.2 Kit/PI(3)K interactions maintain the homeostasis of (a) mast cell subset(s)

Genetic mutation of Y567 and Y569 to phenylalanine (Kit^{FF}mice) within the juxtamembrane of the Kit receptor prevents the binding of Src family protein tyrosine kinase (and other interacting partners such as the tyrosine phosphatases SHP-1 and SHP-2). Kit^{FF} mice have a severe reduction in all mast cell populations

including those within the peritoneum, skin and gastrointestinal tract (Kimura, Jones et al. 2004). The juxtamembrane region of the Kit receptor is often referred to as the regulatory site as such mutations within this region severely attenuate receptor tyrosine phosphorylation and can effect the binding of all SH2 domain containing proteins including p85.

In contrast genetic mutation which *selectively* interfere with Kit/PI(3)K interactions either through site-directed mutagenesis of the PI(3)K binding site, Y719 (Kit^{Y719F}/Kit^{Y719F} mice) or by deletion of p85α lead to tissue site-selective loss of mast cell populations, thus Kit/PI(3)K interactions maintain the homeostasis of (a) specific mast cell subset(s) within the peritoneum and the gastrointestinal tract (Kissel, Timokhina et al. 2000; Fukao, Yamada et al. 2002).

1.7.3 Two convergent pathways control Kit-dependent mast cell homeostasis

Two pathways which maintain Kit-dependent mast cell homeostasis are the PI(3)K and Jun N-terminal kinase (JNK) pathways. Overall molecular and genetic work indicates that the JNK pathway is critical for SCF-dependent proliferation but dispensable for protection from apoptosis which is under the control of the PI(3)K/PKB pathway (Timokhina, Kissel et al. 1998; Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002).

Data from cells expressing mutant Kit receptors which can no longer interact with Src PTK (Kit^{Y567F}), PI(3)K (Kit^{Y719F}) or both (Kit^{Y567F}/Kit^{Y719F}) have shown that each of these pathways which partially contribute towards SCF-induced proliferation and protection from apoptosis converge to activate (via Rac) JNK (Timokhina, Kissel et al. 1998). A more direct interference with the JNK pathway by expressing dominant negative Rac1 (RacN17) or JNK confirms these data but indicates that the JNK pathway does not have a role in SCF-dependent protection from apoptosis (Timokhina, Kissel et al. 1998).

The importance of PI(3)K to SCF-induced proliferation and protection from apoptosis has also been confirmed by gene-targeting. Genetic mutation of tyrosine-719 leads to complete abrogation of Kit associated PI(3)K and a concomitant reduction in PKB phosphorylation. BMMC derived from Kit^{Y719F}/Kit^{Y719F} mice have a substantial reduction (40-60%) in SCF-induced proliferation and protection from apoptosis. Analysis of signalling pathways confirms that PI(3)K activity through Y719 is important for JNK activity (which is reduced by 90%) (Kissel, Timokhina et al. 2000).

Gene-targeted deletion of regulatory adaptors prevents PI(3)K recruitment to activated receptors. Genetic deletion of gene-products encoded by *PIK3r1* indicates the Kit receptor is dependent upon PI(3)K to drive both proliferation and survival. These data also indicate that the p85α regulatory subunit may have additional features utilised by the Kit receptor which are independent of PI(3)-*kinase* activity.

SCF-stimulation of BMMCs derived from p85α null mice (in which p55α and p50α remain intact) have a substantial reduction in JNK activity (60% reduction) but only a minor reduction in PKB phosphorylation (30% reduction), these signalling defects are thought to explain the severe reduction in proliferation (a consequence of a reduction in JNK activity) in the absence of a significant impact on SCF-dependent protection from apoptosis (consequence of a minor defect in PKB) (Fukao, Yamada et al. 2002). These conclusions are strengthened by data from a separate study which utilised mast cells derived from a pan-p85α KO (lacking all *PIK3r1* gene-products) which reported a similar reduction in SCF-induced proliferation as well as a reduction in SCF-dependent protection from apoptosis which correlated with the more severe attenuation of PKB phosphorylation (a consequence of a more severe disruption of Kit associated PI(3)K activity) (Lu-Kuo, Fruman et al. 2000).

These data indicate that the JNK and PKB pathways may have a differential sensitive to perturbations in PI(3)K activity. Attenuation of JNK pathway (critical for SCF-induced proliferation) requires only a minor perturbation of PI(3)K activity whereas in contrast the PKB pathway (which maintains SCF-dependent mast cell survival) requires less PI(3)K input and therefore requires a more robust disruption of PI(3)-kinase activity.

It is also possible that the JNK pathway may be particularly sensitive to deletion of the full length p85α which has unique properties including a N-terminal BH domain

able to interact with Rho family GTPase such as Rac. The nature and the physiological relevance of these interactions are unclear (Deane and Fruman 2004).

The importance of Rac to SCF-dependent responses has been shown by genetic deletion of the haematopoietic restricted Rac2 GTPase. Rac2 null mast cells have a profound attenuation of SCF-induced proliferation, survival, and migration. Rac2 null mast cells also have a complete abrogation of PKB phosphorylation indicative that loss of Rac2 leads to a profound inhibition of PI(3)K activity. This genetic study places Rac upstream of or parallel to PI(3)K(Yang, Kapur et al. 2000).

Kit^{Y719F}/Kit^{Y719F} BMMCs have a 90% reduction in JNK activity (Kissel, Timokhina et al. 2000). The disruption of Y719 in Kit^{Y719F}/Kit^{Y719F} BMMCs leads to abrogation of p85α and p85β association with the Kit receptor. It is therefore possible that this mutation completely abrogates all p85-dependent Rac interactions with the Kit receptor. Deletion of p85α-only (one of two regulatory subunits with a BH domain) reflects a minor perturbation of receptor associated PI(3)K activity (30% reduction in PKB phosphorylation) but it represents a substantial reduction in a Rac interacting partner. The impact of this disruption is a significant (but not total) reduction in (Rac dependent?) JNK (60% reduction) activity (Fukao, Yamada et al. 2002; Tan, Yazicioglu et al. 2003). The remaining PI(3)K-dependent JNK activity (which can be reduced up-to 90% by mutating Y719) maybe mediated through p85β.

Overall the data indicate that the direct PI(3)K binding site on the Kit receptor may be more than a mechanism for activating PI(3)K it may also act as a mechanism to allow Kit to interact with (via p85α and p85β) the Rac GTPase and thereby activate the JNK pathway. It is also possible that PI(3)K may contribute to Rac activation by recruiting PI(3)K-dependent guanine nucleotide exchange factors (GEFs) which may augment Rac activation. A potential model for these interactions is summarised in Fig 1.7.

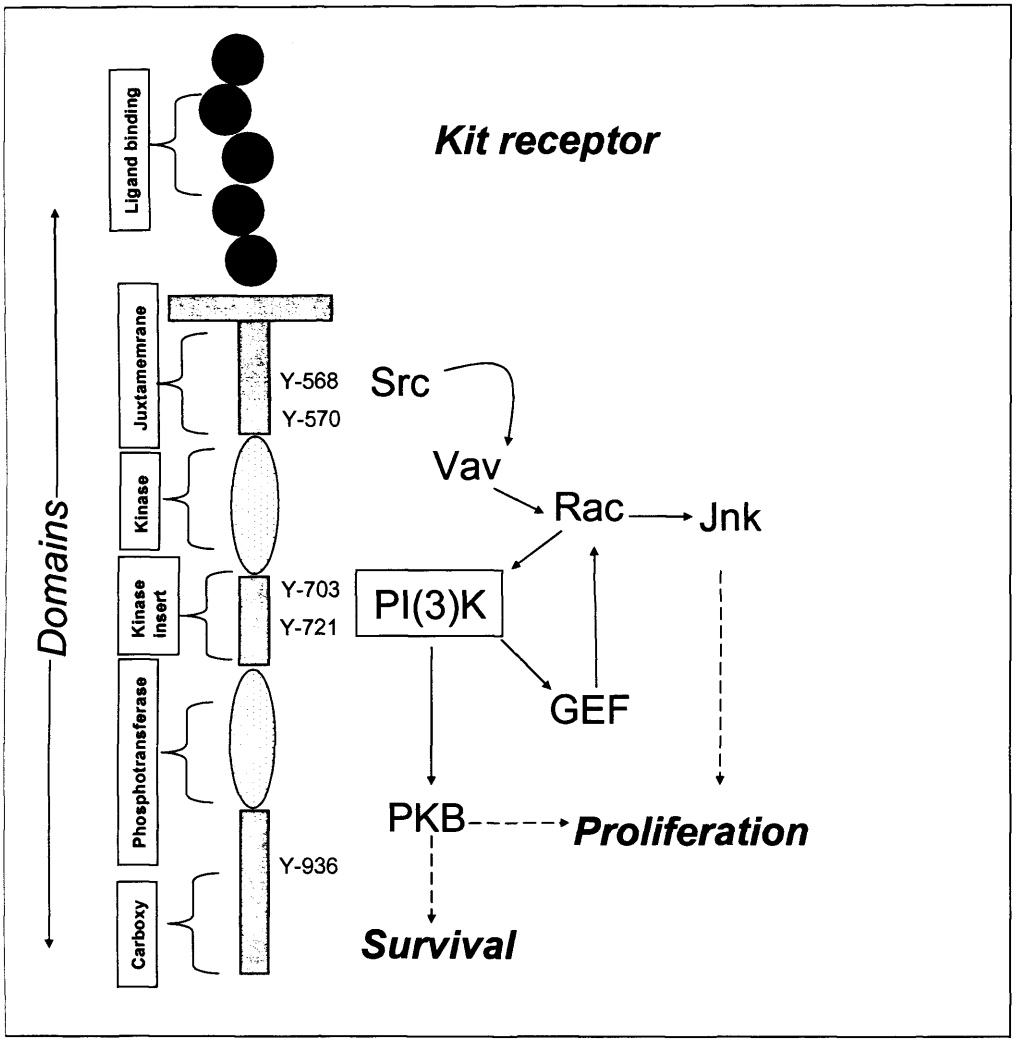


Fig 1.7 Kit receptor-associated PI(3)K activation. PI(3)K/Rac interactions drive mitogenic and survival signals downstream of the Kit receptor.

1.7.4 Kit can recruit Gab2 adaptor associated PI(3)K activity

Interactions between PI(3)K and Kit can be direct and indirect, the latter for example via adaptor proteins such as Gab2 which contain multiple YxxM motifs and can recruit a large quantity of PI(3)K activity to receptor complexes. These associations provide receptors access to a larger pool of PI(3)K than the direct binding sites. Loss

of Gab2 leads to similar *in vivo* mast cell tissue distribution defects to those reported in the p85α KO (Gu, Saito et al. 2001; Nishida, Wang et al. 2002). BMMCs derived from the Gab2 null mice have a partial defect in SCF-induced proliferation (only at higher concentrations of SCF) and PKB phosphorylation (Nishida, Wang et al. 2002).

The data from Kit^{Y719F}/Kit^{Y719F} BMMCs suggest that mutation of Y719 may lead to complete abrogation of PI(3)K association with the Kit receptor including that of Gab2 (although this has not been formally tested). These data can be reconciled if Kit receptor-associated PI(3)K activity consists of two temporally distinct phases, the first 'wave' of PI(3)K activity recruiting the second which maybe coupled to adaptors such as Gab2 which contains a PH domain able to interact with PIP_3 (Fig 1.8). However this hypothesis requires qualification. As there is no detailed kinetic analysis of SCF-stimulated PI(3)K association or PKB activation (assessed only after 5 min) in the BMMC derived from Kit^{Y719F}/Kit^{Y719F}, it remains possible that this mutation does not block the Gab2-recruited PI(3)K activity which may have been recruited at later time-points.

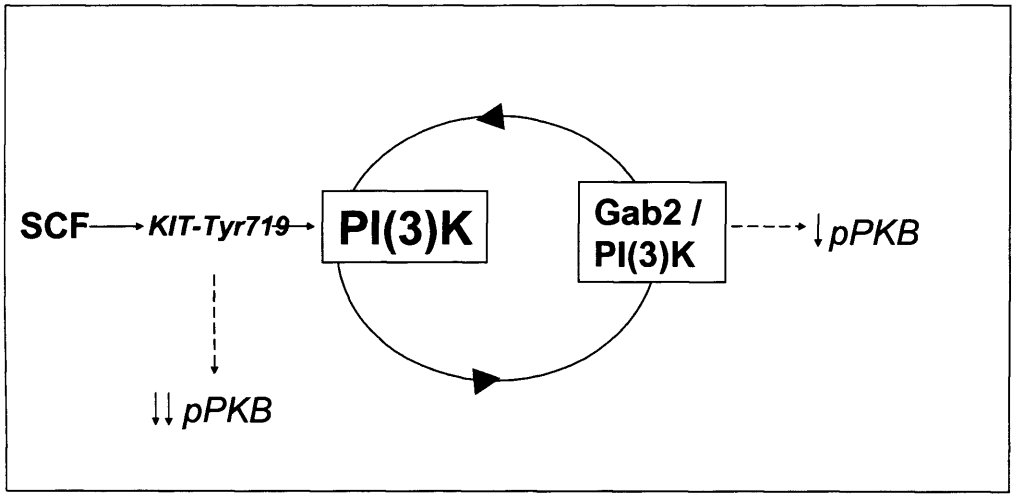


Fig 1.8 PI(3)K dependent recruitment of Gab2-associated PI(3)K activity. *The initial 'wave' of Y719 associated PI(3)K activity may recruit a second phase of Gab2-associated PI(3)K activity to augment Kit-dependent responses. Arrows indicate the impact on PKB phosphorylation of genetic mutation of the direct PI(3)K binding site (Kit-tyrosine 719) or deletion of the Gab2 adaptor protein.*

Table 1.4 Role of PI(3)K in growth factor responses which maintain mast cell homeostasis.

		SCF/c-kit				Interleukin-3			
Targeted subunit	*PI3K activity*	*PI3K activity*	*Proliferation*	*Migration*	*Adhesion*	*PI3K activity*	*Proliferation*	*Tissue Mast cells*	*Reference*
p85α, p50α, p55α	↓ pan -p85 associated ↓ p85β associated	↓ pY ↓ pAkt	↓↓ G1 Arrest	↓↓	-	-	OK	-	(Lu-Kuo, Fruman et al. 2000; Tan, Yazicioglu et al. 2003)
p85α	-	↓ pAkt	↓↓	-	-	-	OK	↓	(Fukao, Yamada et al. 2002)
p85β	-	-	-	-	-	-	-	-	-
P110δ KI	↓↓ YxxM-phosphopeptide ↓↓ p110δ associated	↓ PIP_3 ↓↓pAkt	↓↓	↓↓	↓↓	↓pAkt	↓	↓	(Ali, Bilancio et al. 2004)
P110γ	↓ PIP_3 : GPCR stimulated	OK	-	-	-	OK	-	OK	(Laffargue, Calvez et al. 2002)

- : Not reported
OK : Measured but not affected
↓ : Partial reduction
↓↓ : Substantially reduced / Abrogated

1.8 Role of PI(3)K in FcεRI mast cell activation

Mast cell activation leads to a co-coordinated cascade of events culminating in the exocytosis of preformed and newly synthesised mediators. IgE-dependent antigen-specific responses are directed through FcεRI, a heterotetrameric immunoglobulin receptor composed of (1) a ligand binding FcεRIα chain which binds IgE, (2) a signal amplifying FcεRIβ chain containing two immunoreceptor tyrosine-based activation motifs (ITAMs) and (3) two disulphide-linked FcεRIγ chains critical to signal propagation (Kinet 1999; Rivera, Cordero et al. 2002; Blank and Rivera 2004).

Receptor aggregation initiates a protein tyrosine kinase signalling cascade leading to the activation of two parallel pathways within distinct intracellular compartments, nucleated by the adaptor proteins Linker for activation of T cells (LAT) and Gab2, both of which are known to associate with PI(3)K (Fukao, Terauchi et al. 2003; Blank and Rivera 2004; Deane and Fruman 2004). Cross-talk between these compartments both augments and modulates each pathway and the overall exocytosis process (Blank and Rivera 2004).

1.8.1 Calcium mobilisation and protein kinase C activation are pre-requisite for degranulation

Two pre-requisite events essential for optimal exocytosis and cytokine production are cytosolic calcium mobilisation and activation of protein kinase C (PKC), both of

which known to be influenced by PI(3)K activity (Rivera, Cordero et al. 2002; Fukao, Terauchi et al. 2003; Blank and Rivera 2004; Deane and Fruman 2004).

Receptors that are capable of stimulating the release of intracellular calcium induce the tyrosine phosphorylation of phospholipase Cγ1 (PLCγ1) and PLCγ2, resulting in the generation of the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Zhang, Berenstein et al. 1996; Scharenberg and Kinet 1998). IP_3 binds to its receptor in the membrane of the endoplasmic reticulum and induces the release of intracellular calcium, whereas DAG associates with certain isoforms of the serine/threonine protein kinase C (PKC), promoting their activation (Berridge, Bootman et al. 1998). The IP_3-induced emptying of intracellular calcium stores triggers the entry of extracellular calcium through store-operated calcium channels in the plasma membrane and degranulation (Hoth and Penner 1992; Scharenberg and Kinet 1998).

Degranulation is strictly dependent on the influx of extracellular calcium. Indeed, EGTA chelation of extracellular calcium results in the complete abrogation of antigen-induced mast cell exocytosis (Huber, Helgason et al. 1998). Optimal degranulation also requires inputs other than calcium entry, which is a crucial trigger, but not the only factor responsible for degranulation (Ludowyke, Scurr et al. 1996). The calcium ionophore A23187, can trigger degranulation of mast cells, but less than that elicited by IgE/antigen crosslinking. However, IgE/antigen-triggered degranulation can be mimicked by A23187 in combination with the potent PKC

activator, phorbol ester Phorbol-12-myristate-13-acetate (PMA) (which fails to trigger degranulation in isolation), which suggests that other signals including PKC activation have to be generated for optimal degranulation (Ludowyke, Scurr et al. 1996).

1.8.2 Pharmacological or antibody neutralisation of PI(3)K activity attenuates mast cell exocytosis

1.8.2.1 PI(3)K inhibitors attenuate mast cell exocytosis

Broad spectrum PI(3)K inhibition by LY294002 or wortmannin leads to a profound attenuation of mast cell exocytosis (Yano, Agatsuma et al. 1995; Marquardt, Alongi et al. 1996). Inhibition of calcium flux appears to be partly the mechanism by which wortmannin inhibits degranulation. Wortmannin-treated RBL2H3 cells (a rat mast cell line) have a reduced amplitude and duration of the sustained phase of the calcium response. PI(3)K activation may also be downstream of calcium mobilisation (Huber, Hughes et al. 2000). Thapsigargin is tumor promoter which can induce mast cell degranulation by draining of calcium ions from the endoplasmic reticulum leading to capacitative entry of extracellular calcium through store-operated calcium surface channels, and subsequent degranulation (Bird and Putney 1993; Thastrup, Dawson et al. 1994).

Thapsigargin acts by inhibiting the sacrcoplasmic/endoplasmic reticulum calcium-dependent ATPase, which pumps calcium that leaks from the endoplasmic reticulum back into this organelle (Bird and Putney 1993). Thapsigargin-stimulated degranulation is PI(3)K-dependent and treatment of BMMCs with thapsigargin

transiently activates PI(3)K-dependent PKB (Huber, Hughes et al. 2000). However unlike A23187 and IgE/antigen-induced calcium mobilisation, thapsigargin-stimulated calcium mobilisation is insensitive to PI(3)K inhibitors (Marquardt, Alongi et al. 1996; Huber, Hughes et al. 2000). Thapsigargin-stimulated calcium release is thought to activate PI(3)K which together with PKC can augment the degranulation response.

1.8.2.2 Neutralisation of class IA catalytic subunits attenuates exocytosis

Studies in which neutralising antibodies to class IA subunits have been microinjected into RBL2H3 cells, indicate that all class IA catalytic subunits may participate in exocytosis and are involved in driving both calcium-dependent and -independent pathways (Ching, Hsu et al. 2001; Smith, Surviladze et al. 2001; Windmiller and Backer 2003). However, some functional differences were found between p110α on the one hand, and p110β and p110δ on the other.

Antibody neutralisation of p110β and p110δ led to a reduction in the sustained phase of the calcium response, indicating that these isoforms mediate the wortmannin-sensitive steps in calcium signalling (Smith, Surviladze et al. 2001).

Neutralisation of p110α has no affect on calcium signalling but can still substantially diminish mast cell exocytosis (Windmiller and Backer 2003). The mechanism by which p110α PI(3)K activity contributes to exocytosis is unclear.

There is also evidence to suggest that p110β maybe important in adenosine-potentiated degranulation at suboptimal concentrations of antigen. Neutralisation of

p110β but not p110δ or p110α was found to lead to a substantial attenuation of adenosine-potentiated mast cell degranulation (Windmiller and Backer 2003). The mechanism by which p110β can contribute to this type of response remains unknown.

1.8.3 PI(3)K associates with two parallel pathways activated downstream of FcεRI

There is now a general consensus regarding the signalling pathways downstream of FcεRI. Two parallel pathways are activated following antigen aggregation of FcεRI receptors, namely the Lyn-Syk-LAT and the Fyn-Gab2-PI(3)K axes (Fig 1.9). Electron microscopy suggests that PI(3)K associates with both pathways which are located within different intracellular compartments (Wilson, Pfeiffer et al. 2002). Gene-targeted disruption of the adaptor proteins (LAT or Gab2) which form the nucleus of these pathways leads to profound attenuation of mast cell degranulation and the *in vivo* allergic response. Overall it seems that the Lyn-Syk-LAT and the Fyn-Gab2-PI(3)K pathways have different functions but complement at the level of calcium mobilisation and PKC activation.

1.8.3.1 The Fyn-Gab2-PI(3)K pathway

Antigen aggregation of FcεRI leads to association of the PTK Fyn with the FcεRIβ chain. Fyn phosphorylates Gab2 which then associates with a number of molecules including SHP-2 and PI(3)K. Gab2 null BMMC have an 80% reduction in class IA PI(3)K activity and a substantial attenuation of PKB phosphorylation stimulated by

IgE/antigen. The severe reduction in PI(3)K activity is thought to account for the ~65% reduction in BMMC degranulation *in vitro* and the attenuated *in vivo* IgE/antigen-dependent allergic response. The exact contribution of Gab2/PI(3)K recruited to FcεRI-complexes upon IgE/antigen stimulation is not clear (Rivera, Cordero et al. 2002; Fukao, Terauchi et al. 2003; Blank and Rivera 2004). Apart from the reduced PI(3)K activity IgE/antigen-stimulated Gab2 null BMMC also have a reduction in PLCγ1 tyrosine phosphorylation and a partially reduced calcium flux, although there is some data to suggest that PI(3)K can enhance PLCγ activity (discussed in section 1.8.6) it is not clear whether this reduction in PLCγ1 phosphorylation in Gab2 null cells is a consequence of the reduction in PI(3)K activation or loss of Gab2/PLCγ1 protein-protein interactions (Gu, Saito et al. 2001; Xie, Ambudkar et al. 2002).

It has been suggested that Gab2 associated PI(3)K activity may also influence PKC activation by way of PDK-1, however without direct PKC and PDK-1 activity data this hypothesis requires confirmation. PDK-1 is constitutively active and although it possesses a PH domain there is no data to suggest that PI(3)K activity is important for recruiting or activating PDK-1(Sonnenburg, Gao et al. 2001). PI(3)K activity is more likely to be important for recruiting PDK-1 substrates such as PKB to the membrane where they are phosphorylated (Gu, Saito et al. 2001).

Overall it would seem that Gab2/PI(3)K has functions beyond PLCγ and PKC activation. There are a large number of PI(3)K targets including Tec family protein

tyrosine kinases (such as BTK), GDP-GTP exchange factors (such as P-Rex-1 and cytohesins) and GTPase-activating proteins (including those that activate Arf, centaurin-α or Ras, GAP1^{m}, Gap1^{IP4BP}) some of which may have a role in secretory granule exocytosis(Vanhaesebroeck, Leevers et al. 2001).

1.8.3.2 The Lyn-Syk-LAT pathway

IgE/antigen-stimulation leads to activation of the constitutively FcεRI associated Src family PTK Lyn which phosphorylates tyrosine residues within the FcεRIβ and FcεRIγ chains leading to recruitment of additional Lyn and the Syk PTK which signals downstream of FcεRIγ chain (Kinet 1999; Blank and Rivera 2004). The recruitment of Syk is associated with phosphorylation of the LAT adaptor protein and the formation of a signalling complex termed a 'signalosome' which is primarily concerned with calcium mobilisation and PKC activation. LAT associates with a large number of molecules including PLCγ, adaptor proteins (such as Gads, SLP-76, Grb2) GDP-GTP exchange factors (such as Vav) GTPases (Rac) and PI(3)K (Blank and Rivera 2004). Deletion of LAT leads to reduced activation of many of these signalling proteins and LAT-deficient mast cells have a severe attenuation of calcium signalling, degranulation and cytokine release upon IgE/antigen-activation (Saitoh, Arudchandran et al. 2000). Many of the signalling proteins which associate with LAT augment the activity of PLCγ which is the principal driving force behind mast cell calcium mobililsation (Blank and Rivera 2004).

The downstream targets of PI(3)K activity within the Lyn-Syk-LAT pathway are not clear. Brutons tyrosine kinase (BTK) has been identified as one of the downstream targets of PI(3)K activity which associates with the LAT pathway and can influence PLCγ activation (Hata, Kawakami et al. 1998). BMMC which lack BTK or have a mutation within the BTK PH domain have a partial attenuation of IgE/antigen stimulated calcium flux and the degranulation response (Hata, Kawakami et al. 1998). Few other PI(3)K downstream targets have been identified, currently there is more data available on proteins which can activate PI(3)K including Vav1 and Rac2. Genetic deletion of these PI(3)K 'activating' molecules often leads to a reduction in IgE/antigen-stimulated PI(3)K activity, calcium flux and mast cell degranulation, however as many of these proteins also activate a range of other molecular targets it is not clear whether the reduction in PI(3)K activity in BMMC derived from these mice is directly responsible for the defects in mast cell responses, or whether these deletions have also disrupted other molecular interactions which maybe more important for mobilising calcium and the exocytosis process. (Pivniouk, Martin et al. 1999; Manetz, Gonzalez-Espinosa et al. 2001) (Yang, Kapur et al. 2000).

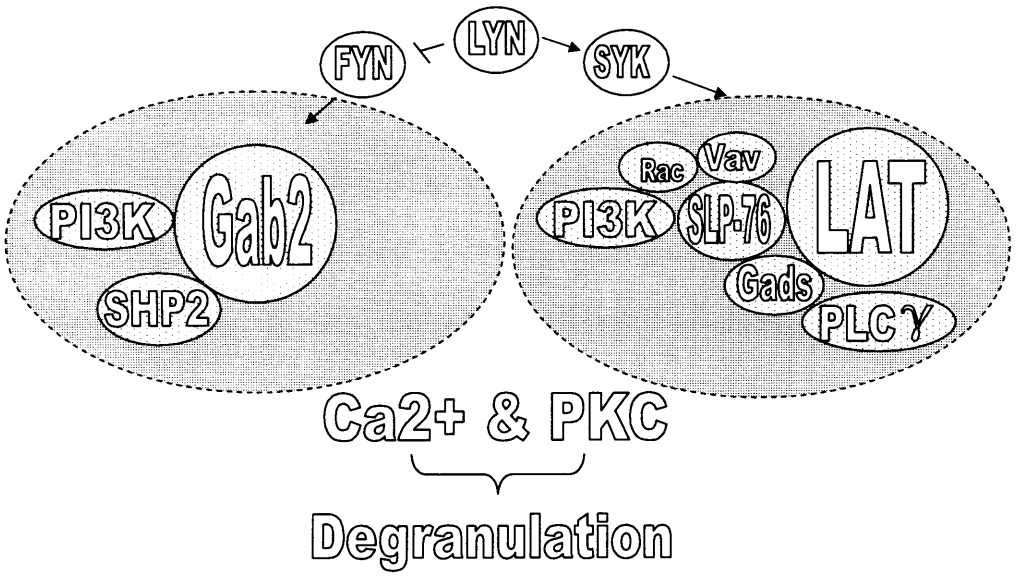


Fig 1.9. Pathways to mast cell degranulation downstream of the FcεRI receptor

1.8.4 Class IA PI(3)K regulatory subunits appear to be not required for mast cell exocytosis

Gene targeting allows for a more focused study of PI(3)K activation downstream of FcεRI. Loss of class IA p85 regulatory subunits through partial or complete disruption of *PIK3r1* or *PIK3r2* has no impact on calcium mobilisation, *in vitro* mast cell exocytosis or *in vivo* anaphylaxis (Table 5) (Lu-Kuo, Fruman et al. 2000; Fukao, Terauchi et al. 2003; Tkaczyk, Beaven et al. 2003). In one report IgE/antigen stimulated PKB phosphorylation was *enhanced* (in pan-p85α null mast cells) and in another kinetically altered (in p85β null mast cells) suggesting that PI(3)K activity was certainly *not* reduced (Table 5) (Lu-Kuo, Fruman et al. 2000; Tkaczyk, Beaven et al. 2003).

The observed increase in PI(3)K (in the pan-p85α KO mast cells) activity is particularly surprising as disruption of the *PIK3r1* has been shown to lead to a down regulation of expression all class IA p110 subunits (Lu-Kuo, Fruman et al. 2000). Although it is possible that in each of these KO mice the remaining regulatory subunit coupled to p110 isoforms compensate for lack of the targeted subunit and maintain normal mast cell degranulation, it is not clear why conditions of reduced p110 expression would lead to increased PI(3)K activity. A possible mechanism for the increase in PI(3)K activity is loss of p85/PTEN phosphatase interactions a consequence of p85 targeting. The PTEN phosphatase normally modulates PI(3)K activity by removing the 3-phosphate from PIP_3 it has been found that loss of p85α has a knock-on reduction in PTEN activity in embryonic fibroblasts and remains to be investigated in p85 KO BMMC (Ueki, Fruman et al. 2002). It is possible that selection for immortalized p85α KO MEFs has selected for cells lacking PTEN. It is also possible that the compensating PI(3)K activity may not be class IA PI(3)K-mediated but by other classes of PI(3)K.

1.8.5 p110γ is critical for mast cell activation and the allergic immune response

Recent data suggest that p110γ plays an important role in mast cell activation. p110γ is recruited by the adaptors p101 and p84 to GPCR signalling and participates indirectly with FcεRI signalling by way of an autocrine or paracrine loop mechanism. Initial activation of FcεRI leads to liberation of intracellular GPCR

agonists like adenosine, which upon interaction with their cognate receptors on the mast cell surface activate p110γ. Activation of p110γ by this route provides an apparently critical burst of PI(3)K activity which overcomes the negative elements of PI(3)K signalling (SHIP and PTEN) leading to full degranulation. Loss of p110γ leads to a reduction in mast cell responsiveness to GPCR ligands and a reduced *in vitro* degranulation without affecting IgE/antigen activated calcium mobilisation (Laffargue, Calvez et al. 2002; Wymann, Zvelebil et al. 2003; Wymann and Marone 2005). However these *in vitro* mast cell exocytosis defects appear to be dose-dependent. No defects are apparent at low antigen concentrations and the autocrine loop can be overcome with higher concentrations of adenosine. Additionally, p110γ-deficient BMMCs activated with IgE/antigen remain sensitive to PI(3)K inhibitors which suggests that other PI(3)Ks may be involved in this process. Irrespective of these *in vitro* findings, *in vivo* the autocrine loop and p110γ activity appear critical for IgE/antigen-dependent anaphylaxis to which p110γ-deficient mice are resistant (Laffargue, Calvez et al. 2002).

1.8.6 Contribution of PI(3)K isoforms downstream of FcεRI remains controversial

Genetic studies have identified a large number of PI(3)K interacting proteins downstream of IgE/antigen stimulated FcεRI-complexes, these include adaptor proteins (Gab2), GEFs (Vav1), GTPases (Rac2), Src and Tec family PTK (Fyn and Btk respectively) etc, however most of molecules apart from BTK have been found to be upstream or parallel to PI(3)K activation. Thus a *critical* PI(3)K-*dependent*

molecule, which when inactivated/deleted would lead to attenuation of degranulation response similar to that observed upon wortmannin treatment of BMMC, has not yet been identified downstream of FcεRI.

BMMC exocytosis is profoundly inhibited by wortmannin, the degranulation event most sensitive to this inhibitor is calcium flux. To date research has been focused on trying to establish how PI(3)Kcan activate the molecules involved in IgE/antigen-dependent calcium flux. PLCγ contains a PH domain and is essential for calcium mobilisation, there are data to suggest that PI(3)K/PIP_3 can directly activate/increase its enzymatic activity through allosteric mechanisms. There is also data which indicates that PI(3)K can augment the activity of PLCγ which is already tyrosine phosphorylated (Falasca, Logan et al. 1998; Smith, Surviladze et al. 2001). However direct PLCγ activation by PI(3)K is disputed by a more recent study in which it was found that wortmannin pre-treatment of primary human and mouse mast cells (which completely abrogated PKB phosphorylation) failed to inhibit PLCγ activation and the initial phase of the IgE/antigen-stimulated calcium response (Tkaczyk, Beaven et al. 2003). This PLCγ dependent and PI(3)K independent phase of the degranulation response was found to be able to maintain a basal level of wortmannin insensitive IgE/antigen-stimulated mast cell degranulation (20% in mouse mast cells and 50% in human mast cells) (Tkaczyk, Beaven et al. 2003). This study also showed that the latent phase of calcium response is wortmannin sensitive and is necessary for optimal degranulation(Tkaczyk, Beaven et al. 2003). The latent phase of calcium flux correlates with influx of calcium from the extracellular environment,

there is data to suggest that PIP_3 can directly or indirectly via Tec family PTK (such as BTK) activate plasma membrane channels which control calcium influx (Ching, Hsu et al. 2001).

To date most research has focused on the role of PI(3)K in calcium mobilisation despite evidence that PI(3)K may also have other roles. Gab2 null BMMC which have a substantial reduction in IgE/antigen stimulated degranulation do not have a severe attenuation of calcium mobilisation. Furthermore p110α neutralising antibodies which do not attenuate calcium mobilisation can also reduce mast cell exocytosis. These data are indicative that PI(3)K may also have other molecular targets involved in the exocytosis process (some of these are highlighted in section 1.8.3.1)

In summary, although PI(3)K activity appears essential for mast cell activation, the nature of the PI(3)K(s) involved remains controversial. Genetic models lacking class IA regulatory subunits preclude a major role for this family in driving IgE/antigen dependent exocytosis. However incomplete disruption of these regulatory adaptor proteins leaves the possibility of compensation by the remaining adaptor proteins. The involvement of class IA PI(3)K activity is supported by genetic data obtained from the Gab2 KO mouse, which forms a major PI(3)K adaptor protein with multiple YxxM motifs able to bind the p85 subunit and is central to one of two parallel pathways critical for mast cell degranulation (see section 1.8.3). Gab2 null BMMCs have a severe reduction in PI(3)K activity (as assessed by both direct and

indirect means) which correlates with a substantial defect in *in vitro* exocytosis and cytokine production.Gab2 null mice are less sensitive to IgE/antigen-dependent systemic anaphylaxis.

It is also possible that non-class IA PI(3)K may also contribute towards mast cell degranulation and there is genetic data to indicate that the p110γ PI(3)K can contribute by way of an autocrine loop mechanism which is essential for optimal mast cell degranualation.

Table 1.5 Role of PI(3)K in mast cell activation.

		FcεRI (Triggered by IgE/antigen cross-linking)				*In vivo (IgE/Ag)*		
Targeted subunit	*PI3K activity*	*PI3K activity*	*Degranulation*	*Cytokine release*	*Calcium Flux*	*Cutaneous anaphylaxis*	*Systemic anaphylaxis*	*Reference*
p85α, p50α, p55α	↓ pan - p85associated ↓ p85β associated	↑ pAkt	OK	-	-	-	-	(Lu-Kuo, Fruman et al. 2000)
p85α	-	↓ pAkt	OK	↓ TNFα (*in vivo*)	-	-	OK	(Fukao, Yamada et al. 2002), (Tkaczyk, Beaven et al. 2003)
p85β	-	↑ pAkt (Early phase)	OK	-	-	-	-	
p110δ KI	↓↓ YxxM phosphopeptide ↓↓ p110δ associated	↓↓pAkt	↓	↓ TNFα ↓ IL-6	↓	↓	↓	(Ali, Bilancio et al. 2004)
p110γ	↓ PIP_3 : GPCR stimulated	↑ pAkt ↓↓pAkt : IgE/Ag + Adenosine	↓ : IgE/Ag ↓↓ : adenosine + IgE/Ag	-	OK : IgE/Ag ↓ : IgE/Ag + adenosine	↓↓ : Adenosine alone	↓↓	(Laffargue, Calvez et al. 2002)

- : Not reported
OK : Measured but not affected
↓ : Partial reduction
↓↓ : Substantially reduced / Abrogated

Aims and objectives

Many of the fundamental biological responses which are thought to maintain mast cell homeostasis are PI(3)K-dependent, as evidenced by the inhibitory effect of broad spectrum PI(3)K inhibitors which block all 8 members of the extended PI(3)K family. The class IA subset of PI(3)Ks which operates in the context of tyrosine kinase signalling, is thought to drive a significant number of responses accredited to PI(3)K activity. This includes growth factor-dependent proliferation, survival and IgE/antigen-dependent secretory granule exocytosis, responses known to be driven to a large extent by tyrosine kinase signalling. At the start of this work, there were few genetic data which could confirm these claims. Indeed, many of the published reports regarding the role of class IA PI(3)Ks in mast cell biology are from studies which have either used indirect targeting strategies, removing important PI(3)K scaffolding proteins such as Gab2 or have introduced genetic mutations which prevent specific receptor from associating with PI(3)K, such as in the Kit^{Y719F}/Kit^{Y719F} mice. The interpretation of results from these approaches can be complicated by 'knock-on' effects which are a consequence of the loss of protein-protein interactions unrelated to PI(3)K. In addition, mast cells from mice in which members of the class IA regulatory subunit family have been removed has in many cases failed to provide phenotypes consistent with a key positive role of PI(3)K in mast cell biology and function. In fact, in some instances an even evidence of *increased* PI(3)K activity was observed in the absence of regulatory adaptors.

This study therefore aimed to more clearly delineate the role of specific class I PI(3)K catalytic isoforms in mast cell biology. Using a combination of gene targeting and pharmacological approaches, we aimed to assess the contribution of the ***kinase*** activity (independently of any scaffolding functions) of p110δ, a PI(3)K isoform which is mainly expressed in leukocytes. Our objectives are (1) reassess the role of class IA PI(3)-*kinase* activity in mast cell responses including growth factor responses which can influence mast cell homeostasis and its role within the allergic immune response (2) assess any p110δ-isoform specific functions.

2. Materials and Methods

2.1 Mice

p110$\delta^{D910A/D910A}$ mice have been described previously (Okkenhaug, Bilancio et al. 2002) and were backcrossed on a C57BL/6 or Balb/c background for 6-10 generations. Age-matched, 6-10 week old littermate mice were used for all experiments.

2.2 *In vitro* methods

2.2.1 Antibodies and pharmacological inhibitors

Stock solutions of small molecule inhibitors were prepared to a final concentration of 10 mM in dimethylsulfoxide (DMSO) (Sigma) from powdered stocks. In cell-based studies, inhibitors were added 20 min prior to stimulation. In all experiments where pharmacological inhibitors were used, an equivalent volume of DMSO vehicle was used in the control samples.

Commercial sources of antibodies and inhibitors used were as follows: p-Akt/PKB S473, Akt/PKB and , p-JNK Thr183, Thr185 were purchased from Cell Signalling Technology (Beverly, MA); Kit-receptor (C-14), p110β, p110δ, p-ERK Y204 (E-4), Erk were from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin was purchased from Sigma; LY294002 was purchased from Calbiochem.

2.2.2 Mast cell cultures

Total bone marrow samples from the femurs of 3-5 mice were cultured in RPMI 1640 medium supplemented with 10% ultra-low IgG FBS (GIBCO), 10 ng/ml IL-3 (Tebu-bio), 20 ng/ml SCF (Tebu-bio), 2 mM L-glutamine, 100 units/ml penicillin and streptomycin (GIBCO) in a 5% CO_2 atmosphere. Bone marrow progenitors were obtained by flushing isolated femurs with a 25-gauge needle and 2 ml of culture medium without growth factors. Cells were counted and seeded at a $0.5x10^6$ cells/ml. The following day the non-adherent cells were removed and transferred to a new tissue culture flask. Cultures were refed with fresh media and IL-3 and SCF every 2 days for the first 2 weeks and then once a week for the remaining culture period.

BMMCs were harvested after an at least 4 week maturation period and tested for Kit receptor and FcεRI expression by flow cytometry using phycoerythrin-labelled rat anti-Kit (CD117; Pharmingen) or anti-DNP IgE (SPE-7; Molecular Probes) and monoclonal FITC-anti-mouse IgE (Molecular Probes), respectively. BMMCs used for this study were cultured in IL-3 and SCF, unless otherwise indicated, with culture times not exceeding 6 weeks.

2.2.3 Cytokine stimulation, lysis and immunoblotting

2.2.3.1 Cytokine stimulation and lysis

Cells were starved for 24 h in normal culture medium lacking IL-3 and SCF. Following stimulation (at 37°C), the cells were washed twice with ice-cold PBS, and

whole-cell lysates were prepared by lysis of pellets in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 1% (w/v) Triton X-100, 150 mM NaCl, 1 mM Ethylenediamine tetra-acetic acid (EDTA), 1 mM NaF, 1 mM Na_3V0_4, 1 mM phenylmethylsulfonyl fluoride, 0.09 trypsin inhibitor units/ml aprotinin (Sigma), 10 μM leupeptin (Sigma), 0.7 μg/ml pepstatin A (Sigma), 27 mM sodium p-tosyl-L-lysine chloromethyl ketone (Sigma) and 1 mM DTT) for 15 min after which insoluble fractions were removed by centrifugation (4°C) at 14,000 rpm for 15 min. Protein concentration (Bio-Rad protein assay) of the supernatant was determined, followed by addition of sample buffer (5x concentrated: 25% glycerol (v/v), 5% SDS (w/v), 250 mM DTT, 156.25 mM Tris.HCl, pH 6.8) and heating to 100°C for 5 min.

2.2.3.2 Immunoblotting

Proteins were resolved on 8% polyacrylamide-SDS gels (constant voltage, 60V overnight) and transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes by wet transfer (200 mA, 6 h) (Millipore). Following transfer, membranes were incubated in blocking buffer (5% (w/v)) dried skimmed milk in 0.1% (v/v) Tween 20 in phosphate buffered saline (PBS-T) for 1 h at room temperature to saturate the aspecific protein binding sites on the membrane.

For immunoblotting, primary antibodies were prepared in PBS-T containing 0.02% azide (w/v) (for phosphospecific antibodies, bovine serum albumin (BSA) and Tris-buffered saline were used). Membranes were incubated with primary antibodies at room temperature overnight on a rocking platform following which membranes were

washed three times (5 min) with PBS-T. Secondary antibodies, conjugated to horseradish peroxidase (HRP) were diluted 1:5000 in 10 ml of 1% milk in PBS-T and incubated with membranes for 1 h at room temperature. The membrane was then washed three times (5 min) in PBS-T and bound antibody was detected with enhanced chemiluminescence reagent (Amersham Pharmacia).

2.2.4 Lipid kinase assay

2.2.4.1 In vitro PI(3)K lipid kinase assay

In vitro lipid kinase assays were carried out on exponentially growing BMMCs. Cells were washed two times with ice-cold PBS and lysed at 4°C in lysis buffer for 30 min (see section 2.2.2) after which insoluble fractions were removed by centrifugation (4°C) at 14,000 rpm for 15 min. Following determination of protein concentration, immunoprecipitations were performed on 500 μg total cell lysate using PI3K isoform-specific antibodies for 1 h at 4°C under rotation. After incubation, immune complexes were captured on protein A-sepharose beads (20 μl of 1:2 slurry) by incubation for a further 2 h. Precipitates were washed three times with PI(3)K kinase buffer (40 mM Tris.HCl pH 7.4, 200 mM NaCl, 1 mM EGTA) before kinase assays were carried by addition of ATP and Mg^{2+} (1 mM ATP, 2.7 mM M_gCl_2 and 0.23 μCi of [γ-^{32}P]ATP/assay (Amersham)) using 200 μg/ml phosphatidylinositol(4,5)bisphosphate (sonicated for 15 min before use) as a substrate. Kinase reactions were performed in a final volume of 60 μl for 15 min at 37°C after which the reaction was quenched by adding 100 μl 0.1 M HCl, followed by 200 μl of chloroform:methanol (1:1). The mixture was vortexed and the phases

were separated by centrifugation at 13,000 rpm for 2 min. The aqueous phase was discarded and the lower organic phase was washed with 200 µl 1 M HCl:methanol (1:1), by centrifugation (13,000 rpm, 2 min) and the aqueous phase was again discarded. The lower organic phase was spotted onto thin layer Silica Gel-60A plates (Whatman), pretreated with 1% oxalic acid, 1 mM EDTA, 40% methanol. TLC plates were run in propan-1-ol, 2 M acetic acid, 5 M H_3P0_4. (65:35:1). After the TLC run, the plates were allowed to dry and autoradiographed. Images of the radiolabelled lipid products were acquired with a PhosphorImager (BioRad) and signals expressed as arbitrary units. The activity within control IgG immunoprecipitates was subtracted from each immunoprecipitate.

2.2.4.2 Determination of total class IA PI(3)K lipid kinase activity

To assess the total class IA-associated PI3K activity *in vitro*, cell extracts were absorbed on a phosphotyrosine peptide matrix and lipid kinase activity was determined (as described in paragraph 2.2.3.1). The Y_PVPMLG [Y_P = phosphotyrosine] peptide matrix used is based on the class IA PI3K docking sites in the intracellular part of the tyrosine-phosphorylated PDGF receptor and contains the consensus binding motif for the SH2 domains of the regulatory subunits of the class IA PI3Ks.

2.2.4.3 Determination of in vivo PIP_3 levels

In vivo levels of PIP_3 were determined by a time-resolved fluorescence resonance energy transfer ligand displacement assay, as described (Gray, Olsson et al. 2003).

BMMCs were starved overnight culture media without growth factors, stimulated the next day in the same media with 100 ng/ml SCF for 10 min and centrifuged at 4°C, 1200 rpm for 5 min after which pellets were resuspended in 0.5 M TCA and immediately frozen on dry ice.

2.2.5 DNA synthesis and mast cell expansion

Following a 24 h starvation in culture medium without IL-3 and SCF, 10^5 cells in triplicate were incubated in 96-well plates (Costar) with 20 ng/ml IL-3 and/or 20 ng/ml SCF, together with 0.5μCi [^{3}H]-Thymidine (Amersham) and harvested 24-72 h later onto a solid filter using a cell harvester (Perkin Elmer), and counted, following application of scintillate using a 96 well Perkin-Elmer Microbeter counter. For expansion assays BMMC were starved overnight in culture medium without IL-3 and SCF, cells were seeded at $0.5x10^6$ cells/ml in 5 ml medium with 20 ng/ml IL-3 or 20 ng/ml IL-3 and SCF. Viable cell numbers were determined every 24 h for a 96 h period using a Casey counter.

2.2.6 Degranulation and cytokine secretion

Mast cells were washed and re-suspended in Tyrodes buffer (10 mM HEPES, 130 mM NaCl, 6.2 mM D-glucose, 3.0 mM KCl, 1.4 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.1% BSA). Unless otherwise stated, cells (5.10^4/well) were plated in triplicate in 96-well flat bottom plates and stimulated for 60 min with IgE/antigen complex [100 ng/ml anti-DNP IgE and 200 ng/ml DNP/HSA (Sigma)] in a final volume of 110 μl, followed by harvesting of cell supernatant and cell pellets. In some experiments,

cells were sensitized overnight with 1 μg/ml anti-DNP IgE, followed by extensive washes and incubation with DNP/HSA, and further treated as above. Cytokine released into the supernatants was assessed using Quantikine kits (R&D Systems). For measurement of β-hexosaminidase activity, 50 μl of supernatant or lysed cell pellet [lysed in 110 μl using hexadecyltrimethyl-ammonium bromide (HTAB; Sigma)] were incubated with 50 μl 3.7 mM p-nitrophenol-N-acetyl-β-D-glucosaminide for 1 h, followed by quenching of the enzymatic reaction by addition of 100 μl 2 M NaOH and measurement absorbance at 405 nm. Degranulation is expressed as a percentage of total β-hexosaminidase activity in the input cells.

2.2.7 Human mast cell studies

Human mast cells were obtained by culture of peripheral blood $CD34^+$ cells in Stem Pro 34 media (Invitrogen, Calsbad, CA, USA) containing recombinant human IL-3 (30 ng/ml), IL-6 (100 ng/ml) and SCF (100 ng/ml) (Pepro Tech, Rocky Hill, NJ, USA) for 1 week, followed by culture without IL-3 (but with IL-6 and SCF as above) for a further 6-7 weeks as described(Okayama, Tkaczyk et al. 2003). For degranulation studies, cells were starved of cytokines overnight and sensitized with myeloma-derived human IgE (Calbiochem) which was biotinylated. Following rinsing with HEPES buffer (as described in (Okayama, Tkaczyk et al. 2003)) containing 0.04% BSA (Sigma) the cells were plated into 96-well tissue culture plates at 10,000 cells per well (with or without inhibitors) and incubated for 15 min at 37°C. Following addition of antigen and/or SCF, degranulation was allowed to proceed for 30 min. The reaction was terminated by centrifugation (4°C, 1000x g, 5

min) and aliquots removed for β-hexosaminidase assay. The remaining cells were lysed by freeze-thawing and duplicate aliquots of these fractions assayed for β-hexosaminidase content. Release was subsequently calculated as the % of total (lysate + supernatant) β-hexosaminidase present in the supernatant.

2.2.8 Cell adhesion and migration

For adhesion assays, cells starved of cytokines for 24 h were washed and resuspended in culture medium without cytokines. Cells were plated at 5.10^4/well in a 50 µl volume in 96-well plates (Costar) pre-coated overnight with 5 µg/ml human plasma fibronectin (Gibco). Following 30 min stimulation with or without SCF (in 100 µl final volume), non-adherent cells were removed by inverting plates for 15 min. The adherent cells were lysed using hexadecyltri ammonium bromide (HTAB) and β-hexosaminidase activity measured to assess the fraction of adherent cells. Adhesion is expressed as percentage of input. For migration assays, exponentially growing cells were washed and resuspended in Tyrodes buffer. 10^5 cells were seeded in 100 µl in the upper chamber of 8 µm pore diameter transwells (BD Biosciences) with or without 100 ng/ml SCF in Tyrodes buffer in the lower chamber. After a 3-4 h incubation at 37°C and 5% CO_2, the cells in the lower chamber were lysed using HTAB and β-hexosaminidase activity measured to assess the fraction of migrated cells.

2.2.9 Calcium flux

Calcium flux was measured in exponentially growing BMMCs. Cells were re-suspended in Hanks-buffered saline solution containing Ca^{2+}, Mg^{2+} (no additional Ca^{2+} or Mg^{2+} was added)(HBSS, Gibco), 2.5 mM probenicid (Sigma) (to prevent dye exclusion) and pre-incubated with 1 μM Fluo-4 (Molecular Probes) for 20 min at 37°C after which 100 μM brilliant black (ICN Biochemicals) was added (to permit fluorescence visualisation)100 μl ($4x10^5$ cells/well) of cell the solution was plated in quadruplicate into 96-well plates (NUNC) and centrifuged at 1300 rpm for 5 min. Stimulations and fluorescence measurements were carried out using an automated FLIPR I machine (Molecular Devices). BMMC were stimulated with 50 μl of 3x concentrated stimulus and the calcium response was followed (over 500 sec.) by measuring fluorescence emission (excitation 488 nm and emission 520 nm). Results are expressed as normalised fluorescence change over time.

2.3 *In vivo* experiments

2.3.1 Passive cutaneous anaphylaxis

The PCA protocols were adapted from Ref. (Tilley, Wagoner et al. 2000). For PCA in the back skin, mice were lightly anaesthetised and injected intradermally at two dorsal sites with 100 ng of murine monoclonal anti-DNP IgE in 20 μl of saline. 24 h later, the mice were injected intravenously with 100 μg of DNP-albumin in 100 μl 0.5% Evan's blue dye in saline (added to permit visual localization of increased vascular permeability). Control mice were given dye in saline only. After 30 min,

animals were terminally anaesthetized, and biopsies around injection sites taken. Evan's blue dye was extracted by incubation of biopsies in 1 ml formamide at 55°C for 48 h and the anaphylactic reaction quantified by measuring the absorbance of at 620 nm (A620). For PCA in the ear, mice were sensitized in one ear with 50 ng anti-DNP IgE diluted in 20 µl saline and were given a sham saline injection in the other ear. 24 h later, the mice were injected intravenously with 1 mg of DNP-albumin in 100 µl 0.5% Evan's blue dye in saline. Ear punches were taken with a sample corer (5mm, Fine Science tools) and the PCA response was quantified by dye extraction from IgE- and saline-injected ears (as above, but in a 200µl volume). Data are expressed as A620 = A620 of IgE-sensitized ear – A620 of saline-injected ear. IC87114 or 75% PEG200 vehicle was administered *per os* 2 h prior to antigen challenge. IC87114 plasma concentration was determined using LC-MS/MS.

2.3.2 Passive systemic anaphylaxis

Mice were sensitized overnight with 2 µg of monoclonal anti-DNP IgE in 200 µl saline by tail vein injection 24 h later, the mice were terminally anesthetised by intra-peritoneal injection with sodium pentobaribital, 150 mg/kg (Merial) and then injected intravenously with 500 µg of DNP-albumin in 100 µl in saline or saline alone. The experiment was terminated 2 min after challenge and blood samples were taken by cardiac puncture. The PSA response was quantified by measuring serum histamine levels using a competitive histamine immunoassay kit (IBL, Hamburg, Germany).

2.4 Statistical analysis

All *in vitro* data shown are representative experiments (mean ± S.D.) from different BMMC cultures (established from at least littermate 3-5 mice each). Data for *in vitro* experiments were statistically analyzed using a t-test and differences between WT and p110$\delta^{D910A/D910A}$ BMMCs were statistically significant ($p<0.05$) unless otherwise stated. Results from *in vivo* experiments (mean ± S.E.M) were assessed using a Mann-Whitney test with results of analysis and animal numbers presented in the relevant figure legends. Graphpad Prism software was used for all statistical analysis.

3.0 Role of p110δ in mast cell homeostasis

3.1 Summary

Previously published work had shown that class IA PI(3)K activity downstream of the Kit receptor plays a role in maintaining the homeostasis of (a) subset(s) of mast cells, namely the mast cells within the peritoneum and gastrointestinal tract (Kissel, Timokhina et al. 2000; Fukao, Yamada et al. 2002). This most likely relates to the observation that Kit-associated PI(3)K activity is important for SCF-induced mast cell proliferation and survival (Kissel, Timokhina et al. 2000; Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002).

Using a previously described gene-targeted mouse model (Okkenhaug, Bilancio et al. 2002) and a p110δ–selective inhibitor (Sadhu, Dick et al. 2003), we determined the role of the class IA PI3K isoform p110δ in mast cell homeostasis. We found that genetic inactivation of p110δ led to a severe reduction in total cell class IA PI(3)K activity and identified p110δ as the predominant class IA PI(3)K in mast cells. We found that $p110\delta^{D910AD910A}$ bone marrow progenitors supplemented with recombinant IL-3 gave rise to substantially reduced numbers (up to 50%) of BMMCs. Indeed, genetic or pharmacological inhibition of p110δ led to a partial reduction in IL-3- induced mast cell proliferation and expansion. $p110\delta^{D910A/D910A}$ BMMC expansion remained LY294002-sensitive and analysis of PI(3)K signalling pathways confirmed that other PI(3)K(s) can contribute and provide sufficient input to maintain IL-3-induced *in vitro* mast cell homeostasis. The nature of the p110δ

independent PI(3)K input and how it couples to the IL-3 receptor remains to be established.

SCF/Kit interactions are critical for *in vivo* mast cell homeostasis. Mast cells express all class IA PI(3)K subunits but have a critical need for p110δ activity which has an exclusive role downstream of the Kit receptor. Genetic or pharmacological blockade of p110δ activity severely attenuated SCF-induced responses such as proliferation, adhesion and migration.

The *in vivo* consequences of these growth factor defects in p110δ mutant mice are similar to those seen in mice lacking p85α and in mice with a mutation within the Kit receptor which can no longer interact with PI(3)K, namely a tissue site-selective reduction in mast cell populations.

We conclude that mast cell homeostasis is maintained by PI(3)K pathways both dependent and independent of p110δ. Whereas the Kit receptor appears to be particularly sensitive to p110δ inhibition, other cytokine receptors are able to utilise non-p110δ sources of PI(3)K together with PI(3)K-independent pathways (such as the Extracellular-Signal Regulated Kinase (ERK) pathway) to maintain *in vivo* mast cell homeostasis. Our data suggest a differential coupling of growth factor receptors to PI(3)K isoforms. Indeed, whereas the IL-3 receptor appears to be able to couple to one or more PI(3)K isoforms, the Kit receptor exclusively utilises p110δ to drive PI(3)K-dependent responses.

3.2.1Role of p110δ in mast cell growth and differentiation

Mast cell homeostasis is dependent on SCF/Kit interactions and is also under the influence of Th2 cytokines (such as IL-3) derived from the local tissue environment (Metcalfe, Baram et al. 1997). *In vitro* mast cells can be derived from bone marrow progenitors supplemented with growth factors such as IL-3 and SCF.

Broad-spectrum PI(3)K inhibition has been reported to lead to profound attenuation of IL-3- and SCF-induced proliferation of mast cells (Scheid, Lauener et al. 1995; Scheid and Duronio 1998; Lu-Kuo, Fruman et al. 2000; Fox, Crew et al. 2005). When we initiated this work, genetic evidence from mouse gene targeting precluded a role for the class IA PI(3)K system in IL-3-driven mast cell development (Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002). Indeed, bone marrow progenitors from class IA regulatory KO mice, cultured in the presence of IL-3, had been reported to give rise to similar numbers of BMMCs as wild-type cells, with no apparent abnormalities in differentiation (Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002).

Using a similar culture protocol as used in these PI(3)K KO studies, we set out to derive BMMCs from bone marrow progenitors from p110$\delta^{D910A/D910A}$ mice. We found that p110$\delta^{D910AD910A}$ progenitors gave rise to substantially reduced numbers (up to 50% less) of BMMC following a 4-6 week maturation period (Fig 3.1a). We found that WT and p110$\delta^{D910A/D910A}$ BMMCs expressed similar levels of the high affinity receptor for IgE (FcεRI) and the Kit receptor (Fig 3.1b). Furthermore,

morphological appearance of toluidine blue-stained cells was similar in BMMCs from both genotypes (Fig 3.1c).

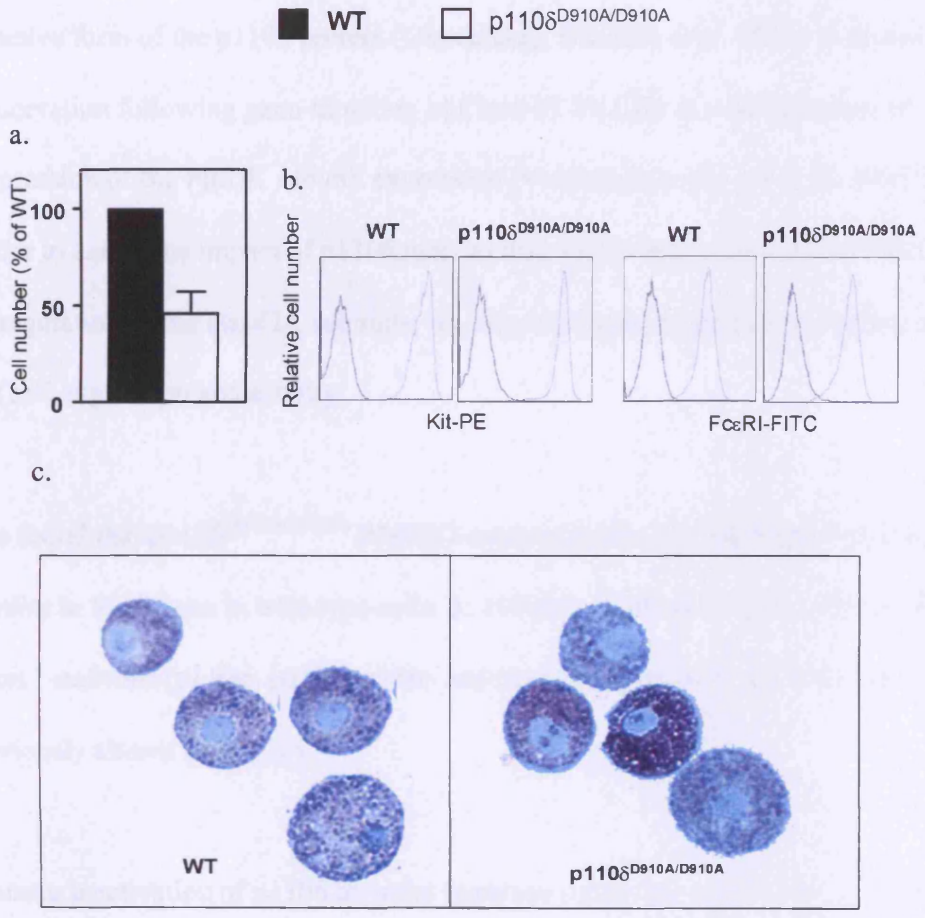


Fig 3.1 p110δ activity is important for *in vitro* growth but not differentiation ***a,*** *Bone marrow progenitor cells were isolated from WT and p110δ$^{D910A/D910A}$ mice and cultured in vitro in the presence of recombinant IL-3 (20 ng ml^{-1}). Following a 4 week maturation period the number of viable cells was assessed. (data is average of 4 independent cultures containing bone marrow from at least 3 mice).* ***b,*** *BMMCs were assessed for mast cell markers. Levels of Kit and FcεRI expression.* ***c,*** *Toluidine blue staining of BMMC from WT and p110δ$^{D910A/D910A}$ mice.*

3.2.2 p110δ is the 'principal' class IA PI(3)K in mast cells

p110δ$^{D910A/D910A}$ BMMCs are derived from mice expressing an enzymatically inactive form of the p110δ protein (Okkenhaug, Bilancio et al. 2002). A common observation following gene-targeting of Class IA PI(3)Ks is a deregulation of expression of the PI(3)K subunit expression (Vanhaesebroeck, Ali et al. 2005). In order to assess the impact of p110δ inactivation, including possible compensation or deregulation of the class IA subunits, we assessed several parameters relating to PI(3)K expression and activity.

We found that p110δ$^{D910A/D910A}$ BMMCs express inactive p110δ protein at levels similar to those seen in wild-type cells. In addition, expression levels of the other class I isoforms (p110α, p110β, p110γ and the p85α regulatory subunit) were not obviously altered (Fig 3.2a).

Genetic inactivation of p110δ by point mutation within the ATP binding site leads to a complete abrogation of p110δ lipid kinase activity in lymphocytes (Okkenhaug, Bilancio et al. 2002). We confirmed this in p110δ$^{D910A/D910A}$ BMMCs by immunoprecipitation (using isoform specific antibodies) of each individual class IA catalytic subunits followed by *in vitro* lipid kinase activity using the physiological substrate PIP_2. Indeed, p110δ activity in p110δ$^{D910A/D910A}$ BMMCs was completely abolished with no alterations in the kinase activities of p110α and p110β (Fig 3.2b). We next assessed the relative contribution of p110δ activity to the overall pool of class IA lipid kinase activity in BMMCs. Total BMMC lysates from WT and

p110δ^{D910AD910A} were incubated with the YpVPMLG peptide (where YP is phosphotyrosine) coupled to a sepharose matrix. PI(3)K complexes isolated using this matrix (which is able to bind all class IA PI(3)K subunits bound to their regulatory adaptors) were then used in *in vitro* kinase assays using PIP_2 as substrate. Total cellular class IA PI(3)K activity in these experiments was reduced by up to 90% in p110δ$^{D910A/D910A}$ BMMCs, indicating that p110δ contributes substantially to the overall class IA PI(3)K activity in these cells (Fig 3.2c).

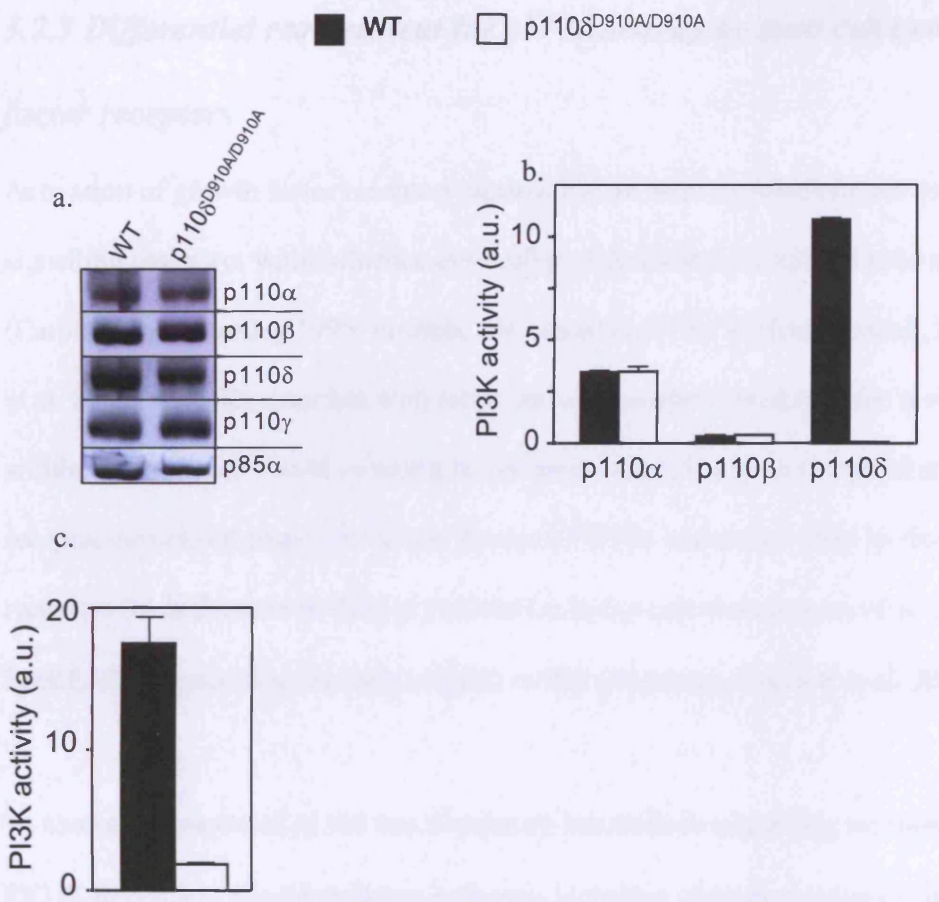


Fig 3.2 p110δ is the principal class IA PI3K isoform in BMMCs *(Experiments were carried out with help from Dr. Antonio Bilancio, Ludwig Institute for Cancer Research, London).* ***a,*** *WT and p110δ$^{D910A/D910A}$ BMMC expression of class IA PI(3)K subunits.* ***b,*** *p110δ lipid kinase activity was assessed following immunoprecipitation of class IA PI3K subunits with isoform-specific antibodies.* ***c,*** *Total class IA lipid kinase activity associated with YpVPMLG (where Yp is phosphotyrosine) peptide complexes.*

3.2.3 Differential requirement for p110δ activity by mast cell growth factor receptors

Activation of growth factor receptors recruits PI(3)K activity which drives important signalling pathways with influence over cell proliferation, survival and activation (Carpenter and Cantley 1996; Fruman, Meyers et al. 1998; Vanhaesebroeck, Leevers et al. 2001). PI(3)Ks associate with receptors via phosphorylated tyrosine residues within the sequence YxxM (where x is any amino acid) found on receptors and receptor-associated adaptor proteins. Receptor PI(3)K association may be direct receptors (as is the case for Kit) or indirect (as in the case downstream of IL-3 and FcεRI) via adaptor proteins such as Gab2 or IRS (Wymann, Zvelebil et al. 2003).

To assess the impact of p110δ inactivation on intracellular signalling we monitored PI(3)K-dependent phosphorylation pathways including phosphorylation of Ser 473 of protein kinase B (PKB, also known as Akt) and Tyr 204 of Erk.

3.2.3.1 PI(3)K signalling downstream of IL-3 is partially p110δ-dependent

We found that IL-3-induced PKB phosphorylation was only partially p110δ-dependent (Fig 3.5). In contrast to PKB, IL-3-induced phosphorylation of Erk was not affected by inactivation of p110δ (Fig 3.3a). In analogy with this genetic inactivation of p110δ, acute inhibition of p110δ in wild-type cells using the p110δ-selective pharmacological inhibitor IC87114 led to a dose-dependent inhibition of PKB phosphorylation following IL-3 stimulation, with a leveling off of the inhibition, leaving a substantial signal of PI3K activation even at the highest doses of IC87114 tested (30 μM, Fig 3.3b). This residual phospho-PKB signal generated in the absence of p110δ activity remained sensitive to treatment with LY294002 and is therefore most likely a result of PI(3)K input, the nature of which remains to be clarified.

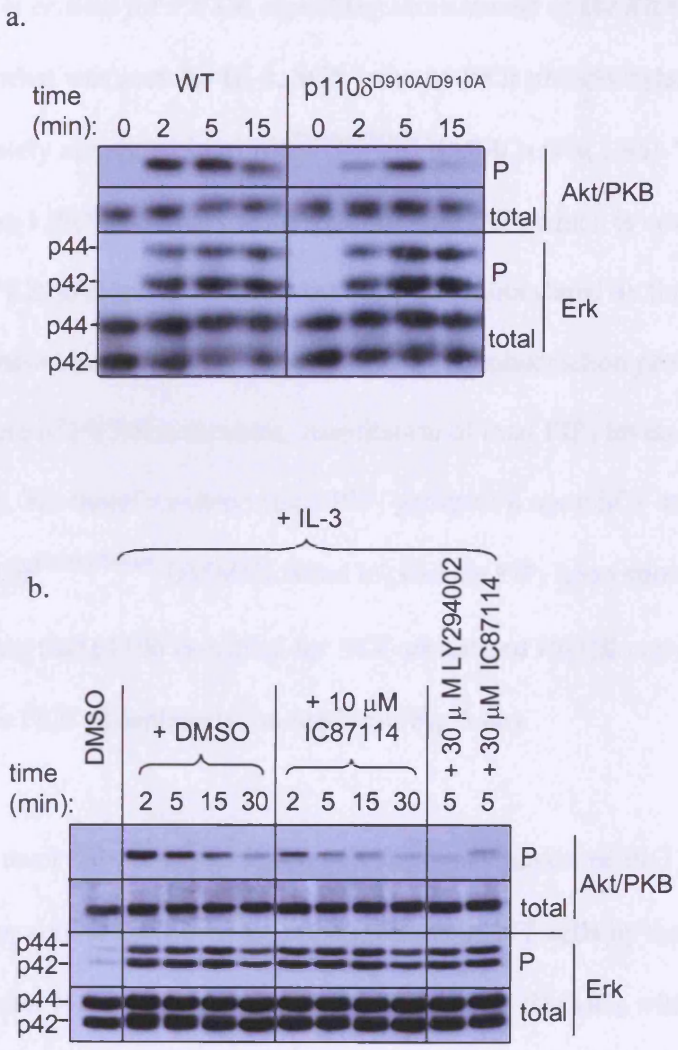


Fig 3.3 IL-3 signalling in BMMCs is partially p110δ-dependent. *(Experiments were carried out with help from Dr. Antonio Bilancio, Ludwig Institute for Cancer Research, London).* ***a,*** *IL-3-induced PKB phosphorylation in p110δ$^{D910A/D910A}$ BMMCs.* ***b,*** *Acute p110δ inactivation in WT BMMC mimics genetic inactivation.*

3.2.3.2 p110δ is critical for PI(3)K signalling downstream of the Kit receptor

In contrast to what was seen for IL-3, SCF-induced PKB phosphorylation was almost completely abrogated in p110δ$^{D910A/D910A}$ BMMCs (Fig 3.4a). The main product of class I PI(3)K activity is the inositol lipid PIP_3 which is critical for recruiting of PKB to membrane sites where it is phosphorylated by the PI(3)K-dependent protein kinase (PDK1). Whereas PKB phosphorylation provides an indirect measure of PI(3)K activation, quantitation of total PIP_3 levels is a more direct measure. We therefore determined PIP_3 generation upon SCF stimulation and found that p110δ$^{D910A/D910A}$ BMMCs failed to produce PIP_3 upon stimulation with SCF, confirming that p110δ is critical for SCF-stimulated PI(3)K activity as revealed by the PKB phosphorylation read-out (Fig 3.4b).

Continuing to use PKB phosphorylation as a surrogate marker of PI(3)K activation we were further able to mimic these genetic defects in WT cells by the p110δ-selective inhibitor IC87114 (Fig 3.5a). Treatment of WT BMMCs with IC87114 led to a dose-dependent reduction in PKB phosphorylation. At doses which are p110δ-selective (5-10 μM), IC87114 could completely abrogate PKB phosphorylation over a prolonged period of time (Fig 3.5b) (Sadhu, Masinovsky et al. 2003). We also tested whether IC87114 has any effect on JNK phosphorylation and report that unlike PKB, phosphorylation of JNK is partially inhibited by IC87114.

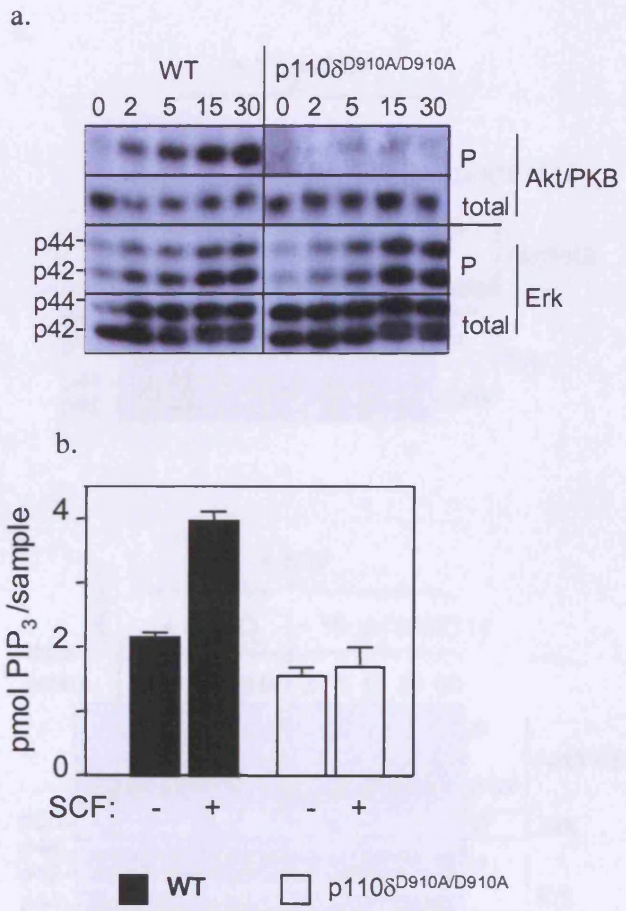


Fig 3.4 p110δ is critical for Kit receptor PI(3)K activity. ***a,*** *WT p110δ$^{D910A/D910A}$ and BMMC stimulated with SCF* ***b,*** *in vivo PIP_3 levels induced by SCF stimulation. The experiment shown in (b) was carried out with help from Dr Alex Gray, Dundee University, UK.*

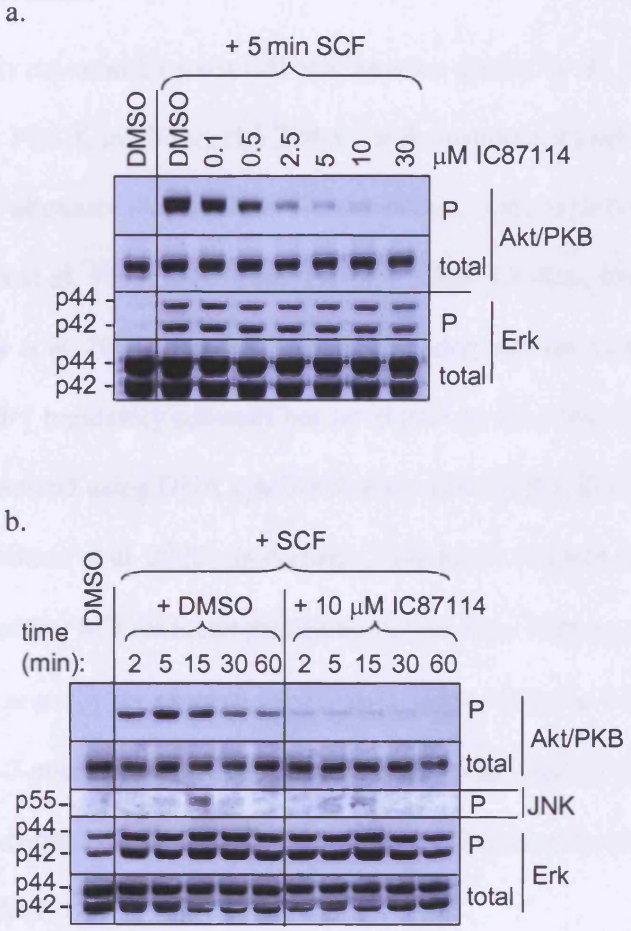


Fig 3.5 Acute pharmacological p110δ inactivation mimics genetic inactivation of p110δ *(experiments were carried out with help from Dr. Antonio Bilancio, Ludwig Institute for Cancer Research, London)* **. a,** *WT BMMCs, pre-treated (30 min) with increasing concentration of IC87114, were stimulated SCF for 5 min.* **b,** *Effect of IC87114 on SCF-induced phosphorylation of PKB and Erk.*

3.2.4 p110δ is essential for mast cell proliferation and expansion

3.2.4.1 Proliferation

PI(3)K activity is essential for mast cell proliferation driven by IL-3 and SCF. Broad-spectrum PI(3)K inhibitors (LY294002 and wortmannin) substantially have been reported to attenuate the response of mast cells to both of these growth factors (Scheid, Lauener et al. 1995; Scheid and Duronio 1998; Lu-Kuo, Fruman et al. 2000; Fox, Crew et al. 2005). Recently two independent studies documented that deletion of *PIK3r1* regulatory subunits has no impact on IL-3-stimulated mast cell proliferation (assessed using DNA synthesis as a measure) (Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002). In contrast, both studies linked PI(3)K coupled to p85α as important for SCF-induced proliferation (Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002). Overall current data suggest that the PI(3)K input which drives IL-3-stimulated proliferation (thought to be class IA PI3K-independent) is distinct from that which promotes SCF induced proliferation (class IA PI3K-dependent).

Using a similar strategy (DNA synthesis) we assessed the contribution of p110δ towards mitogen-stimulated proliferation (Fig 3.6), and found that genetic or acute pharmacological inactivation of p110δ led to a partial block (up to 50%) in DNA synthesis induced by IL-3 (Fig 3.6). In contrast, proliferation induced by ligation of the Kit receptor was found to be almost completely reliant on p110δ activity (Fig 3.6).

Mast cell homeostasis *in vivo* is under the influence of multiple cytokines, with the SCF/Kit interaction being a critical regulator which is further influenced by T cell-derived cytokines within the local tissue environment or released as part of the pathogenic response. Indeed, such Th2 cell factors such as IL-3 can synergise with SCF to promote rapid mast cell proliferation (Metcalfe, Baram et al. 1997). We investigated the co-stimulatory effects of IL-3 in combination with SCF and found that the dramatic synergy in WT cells is substantially attenuated upon p110δ inactivation (Fig 3.6).

Our data indicate that p110δ drives proliferation downstream of both IL-3 and Kit receptors. Inactivation of p110δ compromises the mast cell's ability to proliferate to each factor on its own, a defect which cannot be overcome by co-stimulation with these two important mast cell growth factors.

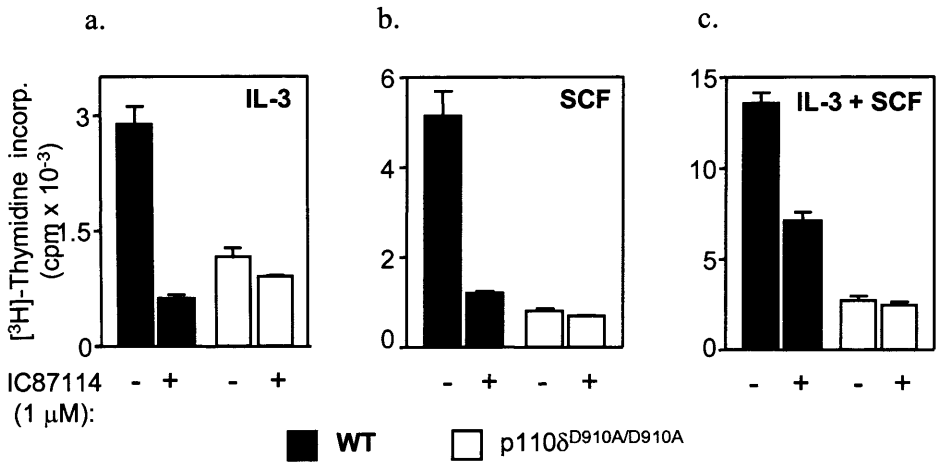


Fig 3.6 p110δ is essential for mitogen-stimulated proliferation. *WT and p110δ$^{D910A/D910A}$ BMMCs were starved of IL3 and SCF for 24 h in complete medium (containing serum), pre-treated (30 min) with or without IC87114 and stimulated with IL3 (**a**) SCF (**b**) or IL-3 and SCF (**c**) for 24h.*

3.2.4.2 Expansion

Mast cells are long-lived cells which retain the ability for expansion following appropriate simulation. *In vitro* generated BMMCs also retain the capacity for cell division over prolonged periods of time.

We found that p110δ$^{D910A/D910A}$ bone-marrow progenitors supplemented with IL-3 gave rise to substantially less BMMCs after a 4-6 week maturation period. Analysis of DNA-synthesis confirmed that part of this defect relates to substantially reduced responsiveness to mast cell growth factors.

We next assessed how the partial defects in proliferation correlate to BMMC expansion. In order to circumvent any unappreciated differentiation defects we assessed this parameter in WT cells stimulated with IL-3 alone, or with IL-3 in combination with SCF, with or without IC87114/LY294002 for 4 days after which the numbers of viable cells were determined. We found that IC87114 (5 μM) could partially attenuate mast cell expansion in the presence of IL-3 alone. Similar to what we observed for proliferative responses, IL-3-stimulated expansion was substantially reduced but not abrogated upon p110δ inactivation.

The capacity of co-stimulation of IL-3 with SCF (more representative of the *in vivo* context where SCF is the primary cytokine which maintains homeostasis by acting as a survival factor which can potently synergise with Th2 cytokines to promote rapid mast cell expansion) to expand cell number was substantially reduced but not abrogated in the presence of IC87114 (Fig 3.7b). We also tested the effect of LY294002 (20 μM) under similar conditions and found that this broad-spectrum PI(3)K inhibitor can completely abrogate mast cell expansion after a 4 day period. This severe disruption of expansion was also associated with a reduction in the number of viable mast cells, indicative of an increase in apoptosis. Remarkably, increased apoptosis was not seen in cells treated with IC87114 (Fig 3.7).

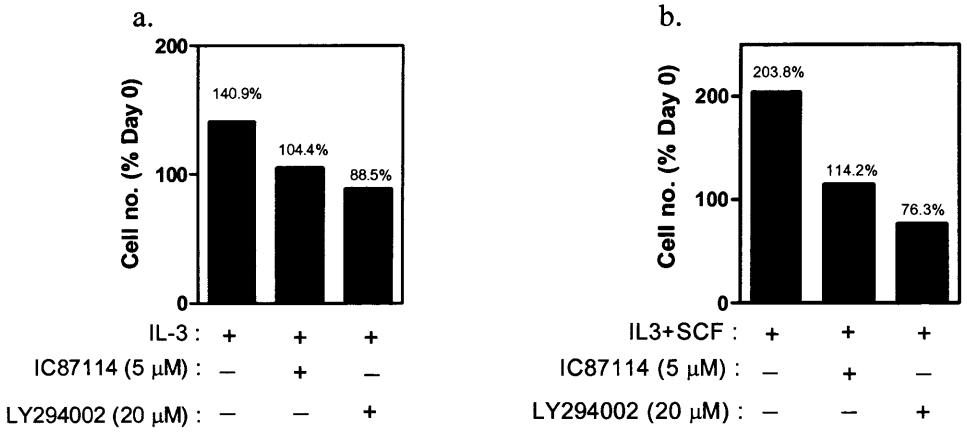


Fig 3.7 Delayed mast cell expansion upon acute inhibition of p110δ. *BMMCs were starved overnight in complete medium (without growth factors but with serum), pre-treated with or without IC87114 / LY294002 (30 min) and stimulated with 20 ng.ml^{-1} IL-3 (a) or 20 ng.ml^{-1}IL-3 and 20 ng.ml^{-1}SCF (b) for 96 h after which the number of viable cells were determined using a Casy counter.*

3.2.5 p110δ is critical for SCF-dependent mast cell adhesion

Mast cells are tissue-resident leukocytes for which adhesion is important to both homeostatic localisation and maintenance in the local environment. Mast cells in the local tissue environment are surrounded by SCF which is important for their survival and proliferation (Metcalfe, Baram et al. 1997). SCF interaction with its receptor Kit enhances mast cell adhesion and is a PI(3)K-dependent process (Dastych and Metcalfe 1994; Serve, Yee et al. 1995; Vosseller, Stella et al. 1997; Tan, Yazicioglu et al. 2003). We investigated mast cell adhesion to fibronectin (as a measure of activity of mast cell integrins) following SCF stimulation. SCF-stimulated BMMCs in which p110δ has been genetically or pharmacologically inactivated exhibited a

substantially diminished ability to adhere to fibronectin. Furthermore no additional impact on adhesion was seen when cells were incubated with LY294002, confirming that the total PI(3)K input in SCF-stimulated adhesion is provided by p110δ (Fig 3.8)

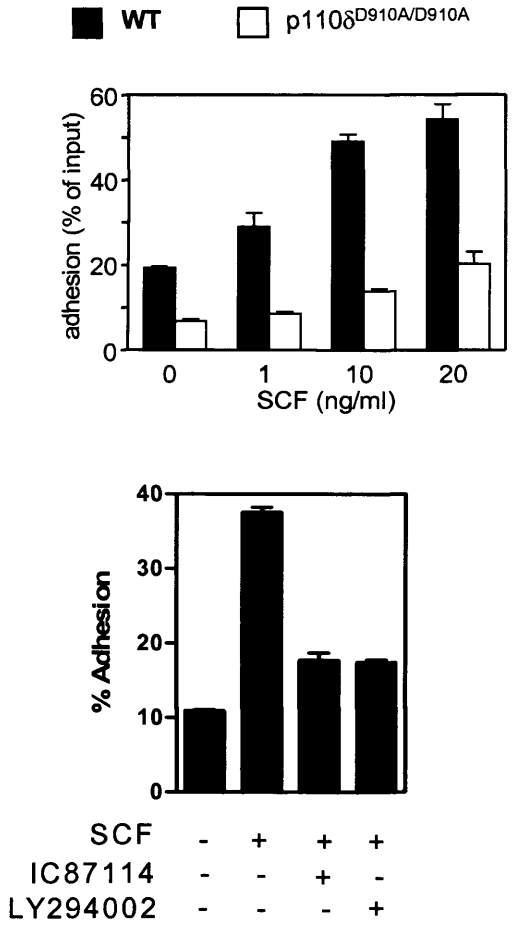


Fig 3.8 SCF-induced mast cell adhesion is p110δ-dependent. ***a,*** *SCF-stimulated adhesion to fibronectin was measured in WT and p110δ$^{D910A/D910A}$ BMMCs. Genetic inactivation of p110δ leads to a substantial defect in basal (unstimulated) and SCF-stimulated cell adhesion.* ***b,*** *Acute inactivation of p110δ in WT cells with IC87114 (5 μM - 30 min pre-incubation).*

3.2.6 p110δ is critical for SCF-dependent mast cell migration

Migration is important for both homeostatic localisation and infiltration to sites of inflammation. SCF is important for correct tissue localisation of mast cell progenitors (Meininger, Yano et al. 1992; Galli, Tsai et al. 1993; Nilsson, Butterfield et al. 1994; Metcalfe, Baram et al. 1997). SCF is known to stimulate mast cell migration (Meininger, Yano et al. 1992). Using a transwell system we assessed mast cell migration towards SCF and found that p110δ$^{D910A/D910A}$ BMMCs have a profound defect in migration towards an SCF gradient (Fig 3.9).

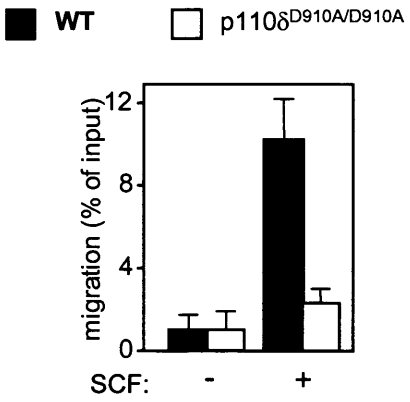


Fig 3.9 SCF-dependent mast cell migration is p110δ-dependent. *Migration of WT and p110δ$^{D910A/D910A}$ BMMCs was assessed in transwell (8 μM) chambers in which BMMCs were placed in the top chamber were allowed to migrate towards an SCF (100 ng.ml^{-1}) gradient in the bottom chamber for 2.5 h. p110δ$^{D910A/D910A}$.*

3.2.7 p110δ^{D910AD910A} mice have site-selective loss of mast cells

Mast cells originate within the bone marrow and are released as immature progenitor cells which migrate and mature within the peripheral tissues (Metcalfe, Baram et al.

1997). We assessed the *in vivo* consequence of constitutive p110δ inactivation on tissue mast cell numbers by histological analysis (Fig 3.1.1 – these experiments were carried out with substantial help from Dr. Nicolas Kuehn at Frimorfo Inc, Fribourg, Switzerland). Mast cell numbers in p110δ$^{D910A/D910A}$ mice were differentially affected in distinct anatomical locations, with defects ranging from severe (peritoneum, jejunum, ileum and colon) over moderate (ear dermis, stomach sub-mucosa and muscularis) to no significant differences (back dermis, mucosa of the stomach) (Table 3.1).

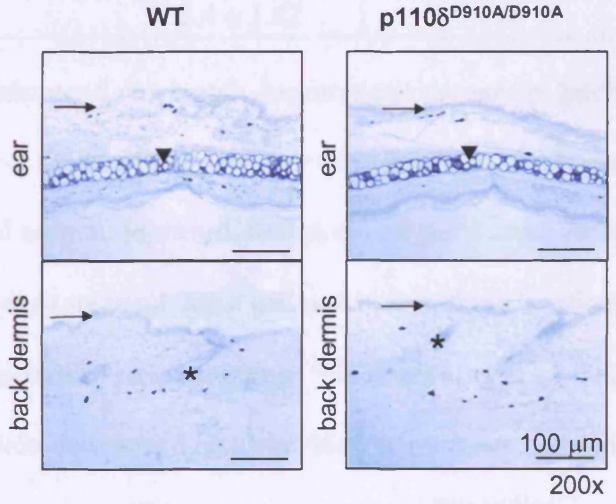


Fig 3.1.1 Site-selective reduction of mast cells in p110δ$^{D910A/D910A}$ mice. *Mast cell numbers were determined in WT and p110δ$^{D910A/D910A}$ mice by staining of tissue sections with toluidine blue). p110δ$^{D910A/D910A}$ mice have reduced numbers of mast cells in the ear dermis but not in the back skin. Arrows, epidermis; arrowheads, cartilage; star, hair follicles with sebaceous glands.*

Table 3.1: Mast cell tissue distribution in WT and p110δ$^{D910A/D910A}$ mice

	Mast cells/unit[a]			
	WT	**p110δ$^{D910A/D910A}$**	**% reduction**	**t-test**
peritoneum[b]	27.7 ± 4.17	2.13 ± 0.76	*92.2*	p<0.001
ear dermis	184.2 ± 35.63	97.17 ± 17.81	*47.2*	p<0.001
back dermis	40.2 ± 3.27	34.2 ± 7.20	*14.2*	ns
stomach: mucosa	67.4 ± 15.69	60.33 ± 23.16	*10.5*	ns
stomach: sub-mucosa	159.6 ± 14.5	91.67 ± 16.54	*42.6*	p<0.001
stomach: muscularis	76.4 ± 12.05	43.5 ± 16.5	*43.1*	p<0.004
Jejunum	4.6 ± 2.07	1.17 ± 1.60	*74.1*	p<0.012
Ileum	3.2 ± 6.1	0.17 ± 0.41	*94.8*	ns
Colon	2.4 ± 1.82	0 ± 0	*100*	p<0.016

[a]Unit: **Ear dermis**: per 5 mm length, beginning at the ear tip; **back dermis:** unit is per 10 high power fields (40x objective = 400x magnification); **stomach:** per one complete sagittal section; **jejunum, ileum, colon:** per 6 cross sections (distance of 50 μm between each section). Mast cell numbers in these locations were determined on toluidine blue-stained tissue sections. [b]Numbers are per 10 fields view under a 200x magnification determined on alcian/blue safranin-stained cells, from cytospins of peritoneal flushes from WT mice (n=3) or p110δ$^{D910A/D910A}$ mice (n=3). Data are presented as the mean ± S.D. from n=5 for WT and n=6 for p110δ$^{D910A/D910A}$ mice.

3.3 Discussion

The primary effector through which PI(3)K can influence mast cell homeostasis appears to be the Kit receptor (Kissel, Timokhina et al. 2000; Fukao, Yamada et al. 2002). Disruption of Kit/PI(3)K associations through mutagenesis of the PI(3)K binding site in the Kit receptor (tyrosine 719) or deletion of the most abundantly expressed class IA regulatory subunit (p85α) has been reported to lead to site-selective loss of mast cell populations, primarily within the peritoneum and gastro-intestinal tract. BMMCs derived from these mice *in vitro* have attenuated SCF-induced responses including proliferation and survival (Kissel, Timokhina et al. 2000; Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002).

A common theme in all of the published PI(3)K genetic models is disruption of expression of the non-targeted subunits, including the catalytic as well as the regulatory subunits (Vanhaesebroeck, Ali et al. 2005). There is now sufficient evidence to suggest that these strategies have disrupted two distinct aspects of PI(3)K biology, namely p110 kinase activity and p85 (and p110) kinase-independent protein-protein interactions. The importance of each of these features to how PI(3)K functions remains to be established. We have focused our efforts to understand the role of PI(3)K p110-kinase functions and have utilised a genetic model in which p110δ expression is maintained but in which the kinase lacks catalytic activity. Our work thus describes a set of phenotypes which can exclusively be ascribed to the kinase activity of p110δ.

BMMCs derived from these p110$\delta^{D910A/D910A}$ mice following a 4-6 week culture period have no obvious differentiation defects and express similar levels of Kit and FcεRI receptors as WT BMMCs. However, we cannot rule out a differentiation defect as we have only assessed a limited number of mast cell markers. A more extensive analysis of receptor and protease expression is required in order to completely rule out any differentiation defects. It might also be of interest to carry out a gene profile analysis for example by array analysis.

We found that p110δ is the principal class IA PI(3)K in mast cells. Indeed, inactivation of p110δ leaves only residual PI(3)K lipid kinase activity towards PIP_2, most likely contributed by p110α and p110β. It is possible that the expression of the latter isoforms is much lower than that of p110δ. Unfortunately the current tools do not allow for accurate quantitation of PI(3)K isoforms. Available antibodies confirm expression of these proteins but cannot easily be used for comparative studies to determine relative levels of each PI(3)K isoform. Each polyclonal antibody used for detecting PI(3)K isoforms is comprised of a mixture of antibodies (directed against a common antigen) derived from B cell clones (directed against different epitope) each with its own affinity for the protein of interest. This difference in affinity is magnified between different polyclonal antibodies and cannot be standardised.

3.3.1 IL-3-associated PI(3)K activity

IL-3-stimulated BMMC proliferation is LY294002 sensitive, however when we initiated this work, there was no direct genetic evidence to suggest the involvement of class IA PI(3)Ks in this response. Indeed, deletion of major class IA regulatory subunits was reported to have no affect on IL-3-induced proliferation or survival (Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002). In contrast, our data indicate that p110δ activity has an important but non-exclusive role downstream of the IL-3 receptor and partially drives the PI(3)K- dependent IL-3-induced proliferation and expansion. Our data are consistent with work in which a dominant-negative class IA PI3K construct (Δp85, which is unable to interact with p110 subunits) was expressed in BMMCs and the IL-3- dependent cell line Baf/3 (Fox, Crew et al. 2005). Expression of Δp85 was found to *delay* cell cycle progression through the G1 phase upon stimulation by IL-3, implicating that class IA PI(3)Ks are important for optimal cell cycle progression in response to this cytokine (Fox, Crew et al. 2005). These data support our own observations that inactivation of p110δ activity delays rather than blocks cell proliferation and expansion. The delay in expansion appears to be modest but amplified over a period of time maybe sufficient to substantially impact mast cell numbers and may explain why we obtain a lower yield of BMMCs after a 4-6 maturation of $p110\delta^{D910A/D910A}$ bone marrow progenitors.

An important question which remains to be answered is the nature of the PI(3)K able to maintain mast cell homeostasis in the absence of p110δ activity. Unlike tyrosine

kinase receptors, the IL-3 receptor recruits PI(3)K activity indirectly via scaffold proteins including Gab2. Thus it maybe that scaffold proteins recruit other class IA PI(3)K isoforms including p110α and p110β, which can maintain *in vitro* mast cell cultures. It is also possible that the IL-3 receptor can recruit other classes of PI(3)K. It is important to mention here that non-PI(3)K pathways, such as the Erk pathway, are unaffected by loss of p110δ activity. These pathways most likely are also critical for mast cell homeostasis *in vivo*.

3.3.2 Kit receptor-associated PI(3)K activity

Our data indicate that p110δ is critical for Kit-receptor-associated PI(3)K activity. Inactivation of p110δ led to a complete abrogation of SCF-induced PIP_3 production and a similar reduction in PKB phosphorylation. The functional consequences of this drastic loss of PI(3)K activity are severe defects in SCF-induced proliferation, adhesion and migration. These findings imply that unlike the IL-3 receptor which can utilise multiple PI(3)Ks, the Kit receptor preferentially utilises p110δ to drive PI(3)K-dependent functional responses. Why then does the Kit receptor not utilise other sources of PI(3)K? It has been shown that Gab2 can recruit to the Kit receptor and provide PI(3)K input which can augment the sustained phase of PI(3)K activity (Nishida, Wang et al. 2002). Loss of Gab2 leads to a partial reduction in SCF-driven proliferation (at high doses of SCF) and a more transient PKB phosphorylation (Nishida, Wang et al. 2002). Like other adaptors that bind PI(3)K, Gab2 has no known selectivity for PI(3)K isoforms and can associate with all class IA PI(3)K subunits. Genetic modification of tyrosine-719 on the Kit receptor has been reported

to lead to complete abrogation of Kit/PI(3)K association and activity (Kissel, Timokhina et al. 2000). As this mutation is not expected to directly affect Gab2 signalling it is surprising that BMMCs derived from Kit^{Y719F}/ Kit^{Y719F} mice do not recruit Gab2-associated PI(3)K. Thus, tyrosine-719-associated PI(3)K appears important for recruiting a second phase of PI(3)K (possibly Gab2-associated) which increases the amplitude and duration of PI(3)K activity. Clearly both phases of PI(3)K activity are important for mast cell homeostasis as both $Kit^{719F/719F}$ and Gab2 KO mice have similar mast cell tissue distribution defects (Fig 3.1.2) (Kissel, Timokhina et al. 2000; Gu, Saito et al. 2001; Nishida, Wang et al. 2002).

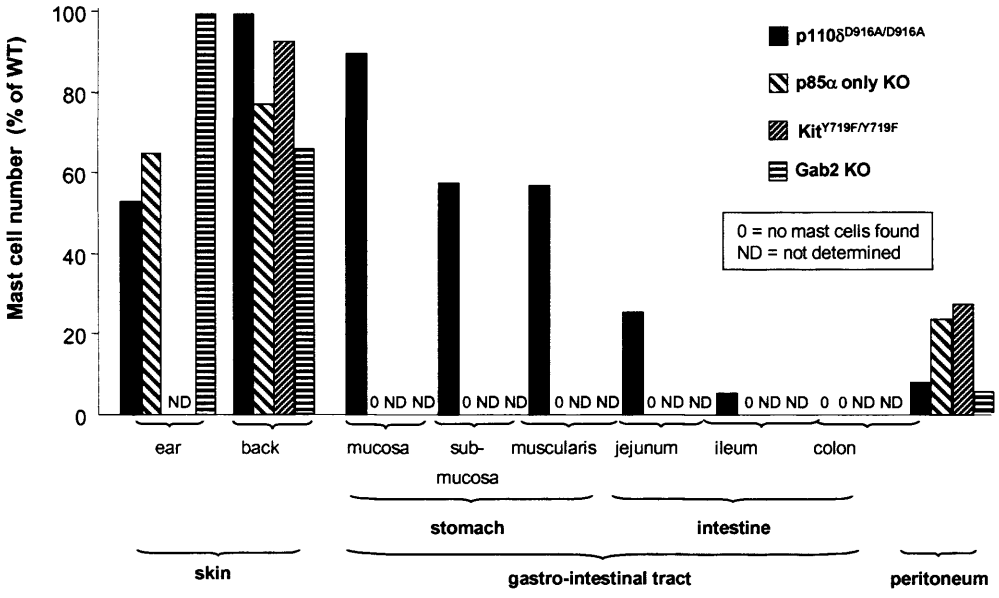


Fig 3.1.2 Comparison of mast cell tissue distribution of different mutant mice. *A comparison of mast cell tissue distribution from mutant mice in which PI(3)K (p85α and p110δ), PI(3)K-receptor binding site (Kit-tyrosine-719) or major PI(3)K scaffold (Gab2) proteins have been gene-targeted.*

3.3.3 Class IA p110δ activity is important for maintaining the homeostasis of a subset of mast cells

The SCF/Kit interaction is the primary signal which maintains mast cell homeostasis *in vivo* (Metcalfe, Baram et al. 1997). Disruption of Kit kinase activity is known to lead to severe reductions in mast cell numbers in all anatomical locations (Metcalfe, Baram et al. 1997). Our data are in line with the hypothesis that PI(3)K activity downstream of the Kit receptor is important for maintaining the homeostasis of a certain mast cell subset(s). Recently it has been shown that the two forms of SCF (membrane-bound SCF, expressed on endothelial cells, fibroblasts, keratinocytes etc) and soluble SCF (cleaved from the surface of endothelial cells and fibroblasts) differentially utilise PI(3)K activity (Trieselmann, Soboloff et al. 2003). Therefore it is possible that mast cells residing in anatomical locations in which mast cell numbers are profoundly reduced, utilise (or have access to) primarily soluble SCF which would make these cells more sensitive to PI(3)K inhibition. We have not tested how BMMCs derived from p110$\delta^{D910AD910A}$ mice respond to membrane-bound SCF. Future areas of investigation a more detailed analysis of *in vivo* mast cell tissue distribution and any differences in how membrane bound and soluble SCF utilise p110δ activity.

4.0 Role of p110δ in the allergic response

4.1 Summary

PI(3)K has an important role downstream of the FcεRI receptor, as shown by the attenuation of degranulation by the broad-spectrum PI(3)K inhibitors LY294002 and wortmannin (Yano, Agatsuma et al. 1995). It was therefore remarkable that cells from mice with deletions in the PI(3)K regulatory subunits had no effect on mast cell degranulation and appeared even to have enhanced PI(3)K signalling pathways under certain conditions, for example downstream of the FcεRI (Lu-Kuo, Fruman et al. 2000; Vanhaesebroeck, Ali et al. 2005).

Using cells derived from p110δ^{D910AD910A} mice and a p110δ-selective small molecule inhibitor, we have assessed the contribution of the p110δ isoform downstream of IgE/antigen-triggered mast cell responses. We found that inactivation of p110δ led to substantial attenuation of PI(3)K-dependent PKB phosphorylation, mast cell degranulation and a reduction in the release of the pro-inflammatory cytokines TNFα and IL-6. In addition, calcium flux (a pre-requisite for optimal degranulation) was substantially reduced in p110δ^{D910AD910A} BMMCs.

Human mast cells, pre-incubated with the p110δ inhibitor IC87114 at a p110δ–selective dose, showed attenuated FcεRI receptor-induced degranulation Furthermore in a more physiological context, IC87114-treated human mast cells

stimulated with a sub-optimal dose of antigen in presence of SCF have a more severe attenuation of the exocytosis response.

p110δ^{D910AD910A} mice had a substantially diminished IgE-dependent passive cutaneous anaphylaxis (PCA) response which could also be mimicked by treating wild-type mice with IC87114 at a dose which is selective for p110δ. The PCA response could be completely blocked at higher doses of IC87114 at which this compound may also block PI(3)K isoforms other than p110δ.

We conclude that p110δ together with other PI(3)K(s) is essential for driving IgE/antigen-dependent mast cell degranulation. Genetic or pharmacological inactivation of p110δ leads to a substantial reduction in mast cell degranulation which at the whole animal level can attenuate the allergic immune response.

4.2 In vitro allergic immune response

4.2.1 p110δ activity is essential for FcεRI signalling

Mast cells respond to specific antigen as part of the allergic response which is directed through the high affinity receptor for IgE (FcεRI) (Metcalfe, Baram et al. 1997; Wedemeyer and Galli 2000; Puxeddu, Piliponsky et al. 2003; Blank and Rivera 2004). The FcεRI binds antigen-specific IgE which responds to antigen cross-linking by activating a series of intracellular cascades leading to degranulation and cytokine release. PI(3)K activity has been identified as being essential for mast cell degranulation (Yano, Agatsuma et al. 1995; Ching, Hsu et al. 2001; Smith,

Surviladze et al. 2001; Windmiller and Backer 2003). PKB phosphorylation on Ser473 or Thr308 serves as an indirect measure of PI(3)K activity and we have shown that this can be used as a reliable measure of PI(3)K status within BMMCs (see section 3.2.3). We found that PKB phosphorylation following antigen cross-linking is substantially reduced upon genetic inactivation of p110δ (Fig 4.1). In contrast, Erk/MAPK phosphorylation was found to be unaltered (Fig. 4.1).

Fig 4.1 p110δ is essential for FcεRI-stimulated PI(3)K activity. *WT and p110δ^{D910AD910A} BMMCs were pre-incubated overnight with monoclonal anti-DNP IgE Clone SPE-7 (1 mg.ml^{-1}) and challenged the following day with antigen (anti-DNP HSA, 100 ng.ml^{-1}) for the indicated times, followed by immunoblotting of cell extracts for the indicated signalling molecules.*

4.2.2 In vitro activation of murine BMMCs

Using an *in vitro* degranulation assay and β-hexosaminidase activity (an intracellular granule-associated enzyme released as part of the exocytosis process) to measure

this response, we assessed the impact of p110δ inactivation on IgE/antigen-triggered specific immune responses in BMMCs (Fig 4.2a). Following sensitisation with antigen-specific IgE (specific for the hapten dinitrophenol), BMMCs were triggered to release intracellular mediators upon subsequent exposure to antigen (dinitrophenol coupled to BSA). Genetic or pharmacological inactivation of p110δ led to a substantial (~40-50%) reduction in the IgE/antigen-triggered mast cell exocytosis response, a defect which is maintained across a broad-range of antigen concentrations tested (Fig 4.2b).

The residual response remaining in $p110\delta^{D910A/D910A}$ BMMCs could be further inhibited to a maximum of 80% by treatment with LY294002 (Fig 4.2a). These findings suggest that there is a LY294002-sensitive pool of PI(3)K which contributes towards the exocytosis process and is responsible for maintaining the partial PI(3)K-dependent degranulation response in $p110\delta^{D910A/D910A}$ BMMCs, accounting for ~35% of the overall degranulation response. The remaining LY294002-insensitive *in vitro* degranulation response (~25% of the total response (Fig4.2a)) appears not to depend on PI(3)K activity and may be a consequence of the activation of other signalling pathways, such as the PLCγ pathway. IC87114 can substantially attenuate the degranulation of WT BMMC but has no additional effects on the exocytosis response of $p110\delta^{D910A/D910A}$ BMMC confirming that this compound is on target (Fig 4.2c).

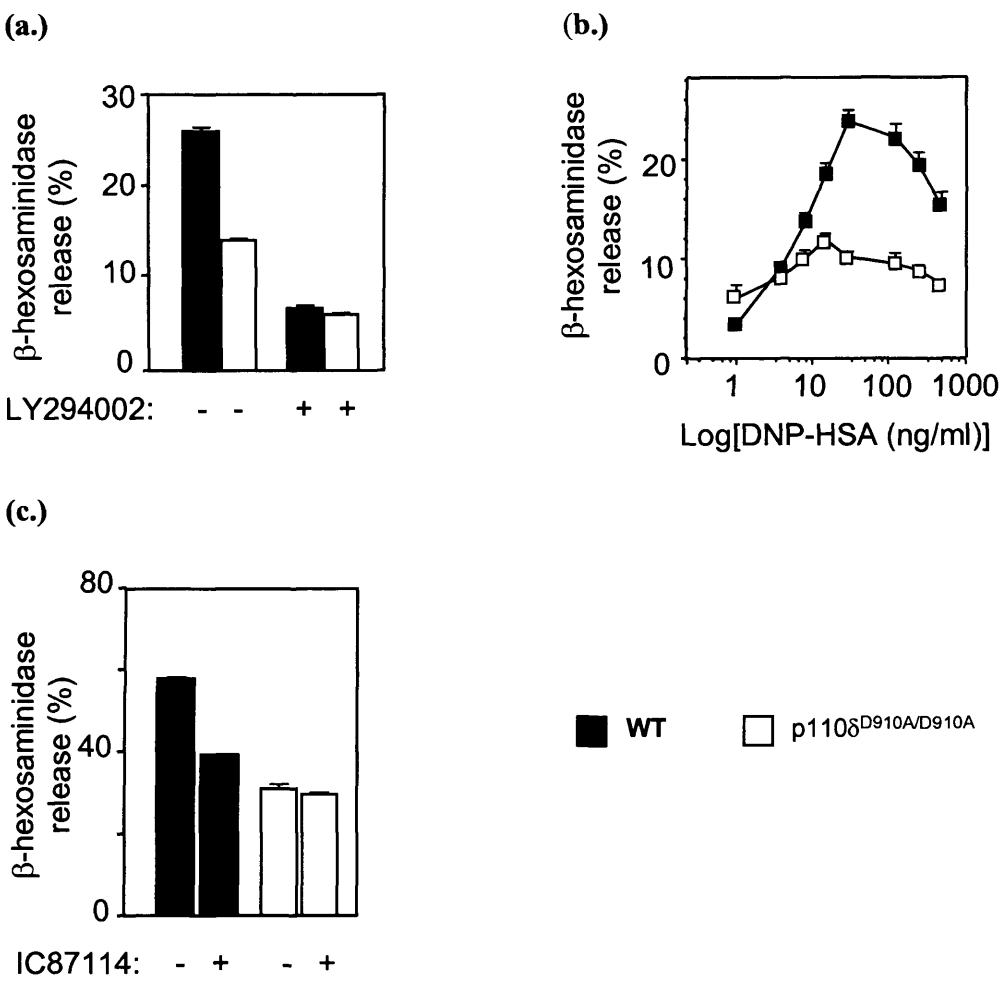


Fig 4.2 p110δ is essential for degranulation of mouse mast cells. ***a,*** *Exocytosis was measured (β-hexosaminidase release from mast cell granules) in exponentially growing WT and p110δ$^{D910A/D910A}$ BMMCs pretreated with or without LY294002 (30 μM for 30 min) and stimulated with IgE/antigen complex. Degranulation is expressed as percent of total cellular β-hexosaminidase.* ***b,*** *WT and p110δ^{D910AD910A} BMMCs pre-incubated with 1 mg.ml^{-1} of IgE were stimulated by varying concentrations of antigen, followed by measurement of β-hexosaminidase release .* ***c,*** *Impact of IC87114 was assessed in WT and p110δ$^{D910A/D910A}$ BMMC which were pre-incubated with or without IC87114 (5 μM).*

4.2.3 Cytokine production by BMMCs

A consequence of IgE/antigen-triggered mast cell degranulation is the release of extracellular cytokines (Galli, Kalesnikoff et al. 2005). We measured the release of TNFα and IL-6, two important pro-inflammatory cytokines, and found that p110δ$^{D910A/D910A}$ BMMCs released substantially reduced amounts of these factors upon IgE/antigen activation (Fig 4.3).

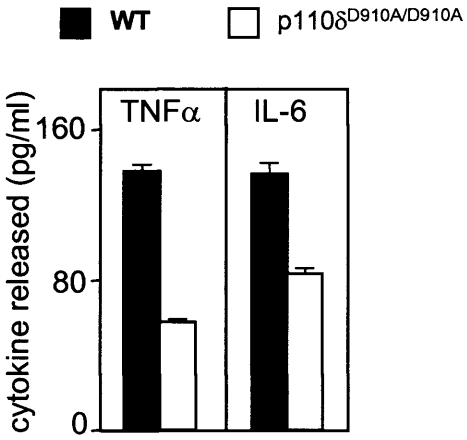


Fig 4.3 Reduced release of pro-inflammatory cytokines upon FcεRI ligation in p110δ$^{D910A/D910A}$ BMMCs. *WT and p110δ^{D910AD910A} BMMCs were activated with IgE/Ag complex for 1 h after which cytokine levels were determined in supernatants.*

4.2.4 Calcium flux in BMMCs

Calcium mobilisation is a prerequisite for the degranulation response and has two distinct phases, namely first calcium release from intracellular stores followed by influx of calcium from the extracellular environment (Yano, Agatsuma et al. 1995; Ching, Hsu et al. 2001; Smith, Surviladze et al. 2001; Windmiller and Backer 2003; Blank and Rivera 2004).

Calcium release following IgE/Ag cross-linking in p110δ$^{D910A/D910A}$ BMMCs was substantially but not completely ablated (Fig 4.4).

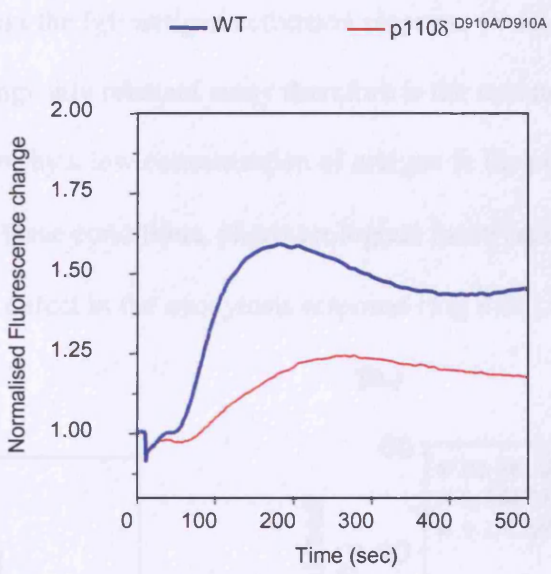


Fig 4.4 p110δ activity is important for IgE/Ag-activated calcium flux in BMMCs. *WT and p110δ^{D910D910A} BMMCs preloaded with Fluo-4 were activated with IgE/Ag in 96-well plates (2 X 10^5 cells per well). Intracellular calcium flux was followed over time by measuring fluorescence emission at 520 nM.*

4.2.5 In vitro activation of human mast cells (experiment carried out by Christine Tkaczyk and Alasdair M. Gilfillan, NIH, USA)

Using IC87114, our collaborators in the US assessed how our data obtained from mouse BMMCs compared with the human system. Similar to what we found in murine BMMCs, acute pharmacological inactivation of p110δ in human mast cells resulted in a ~40% reduction in mast cell degranulation. This attenuated human mast

cell degranulation could be further reduced by treatment with LY294002 (Fig. 4.5a), indicative that multiple PI(3)Ks drive human mast cell degranulation.

In vivo, mast cell activation occurs in an SCF-rich environment. SCF has been shown to augment the IgE/antigen activation response (Vosseller, Stella et al. 1997). A more physiologically relevant assay therefore is the measurement of mast cell activation *in vitro* by a low concentration of antigen in the presence of SCF. It is clear that under these conditions, pharmacological inactivation of p110δ led to a very substantial defect in the exocytosis response (Fig 4.5b).

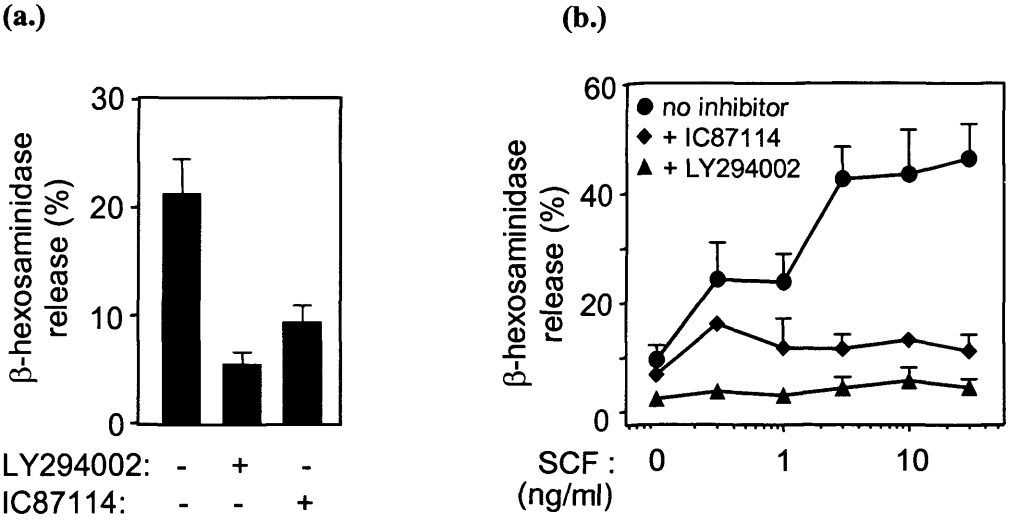


Fig 4.5 p110δ is essential for human mast cell degranulation. a, *Human mast cells were sensitized with IgE overnight and challenged with antigen (1 ng.ml^{-1}) following 30 min pre-incubation with or without LY294002 (30 μM) or IC87114 (5 μM).* ***b****, IgE-sensitized human mast cells, pre-incubated with or without IC87114 (5 μM) or LY294002 (30 μM), were challenged with sub-optimal concentrations of antigen (0.1 ng.ml^{-1}) in the presence of varying does of SCF.*

4.3 In vivo allergic immune responses

4.3.1 Passive cutaneous anaphylaxis

We next investigated the impact of p110δ inactivation on allergic responses in a murine model of anaphylaxis. *In vivo* anaphylaxis through the FcεRI receptor is an allergic response that is predominantly mast-cell-dependent and occurs as a result of either local or systemic exposure to allergens, which crosslink and activate antigen-specific IgE bound to the FcεRI on the mast cell surface (Wedemeyer and Galli 2000). We assessed the capacity of wild-type and p110δ$^{D910A/D910A}$ mice to respond to immune challenge in a passive cutaneous anaphylaxis (PCA) model. Mice were given an intradermal injection of IgE directed against a hapten (dinitrophenyl (DNP) in this case). 24–48 h after this priming event, the mice were challenged by systemic administration of DNP coupled to a carrier protein (HSA) together with Evan's blue dye, which binds to serum proteins. Mediators released upon mast cell activation increase vascular permeability, causing oedema and allowing the dye to leak from the blood vessels into the surrounding tissue. The amount of extravasated Evan's blue provides an indirect measure of mast cell activation. We assessed the PCA response in two distinct tissue locations. In the ear of p110δ$^{D910A/D910A}$ mice, a marked (80%) reduction in PCA was observed. This decrease was more significant than the reduction in mast cell numbers at this location (maximally 47% - Table 3.1), suggesting that the remaining mutant mast cells may be defective.

The PCA reaction in the back dermis, a site with unaltered mast cell numbers in p110$\delta^{D910A/D910A}$ mice (Table 3.1), was also significantly reduced (40%, see Fig 4.5b).

IC87114, at doses at which it is selective for p110δ (plasma concentration of 5 μM from a 15 mg kg^{-1} dose), reduced the allergic response by 35–40% in the back skin and ear of wild-type mice (Fig 4.6). At higher plasma concentrations (40 μM from a 60 $mgkg^{-1}$ dose), IC87114 completely blocked the anaphylaxis reaction in the back dermis (Fig 4.6c). This may be a consequence of the inhibition of other PI(3)K isoforms (such as p110γ), which could account for the remaining PI(3)K-dependent (that is, LY294002-sensitive) degranulation seen in p110$\delta^{D910A/D910A}$ BMMCs (Fig. 4.2a).Taken together, our data show that this class of small molecule inhibitors of PI(3)K can completely abrogate *in vivo* cutaneous anaphylaxis.

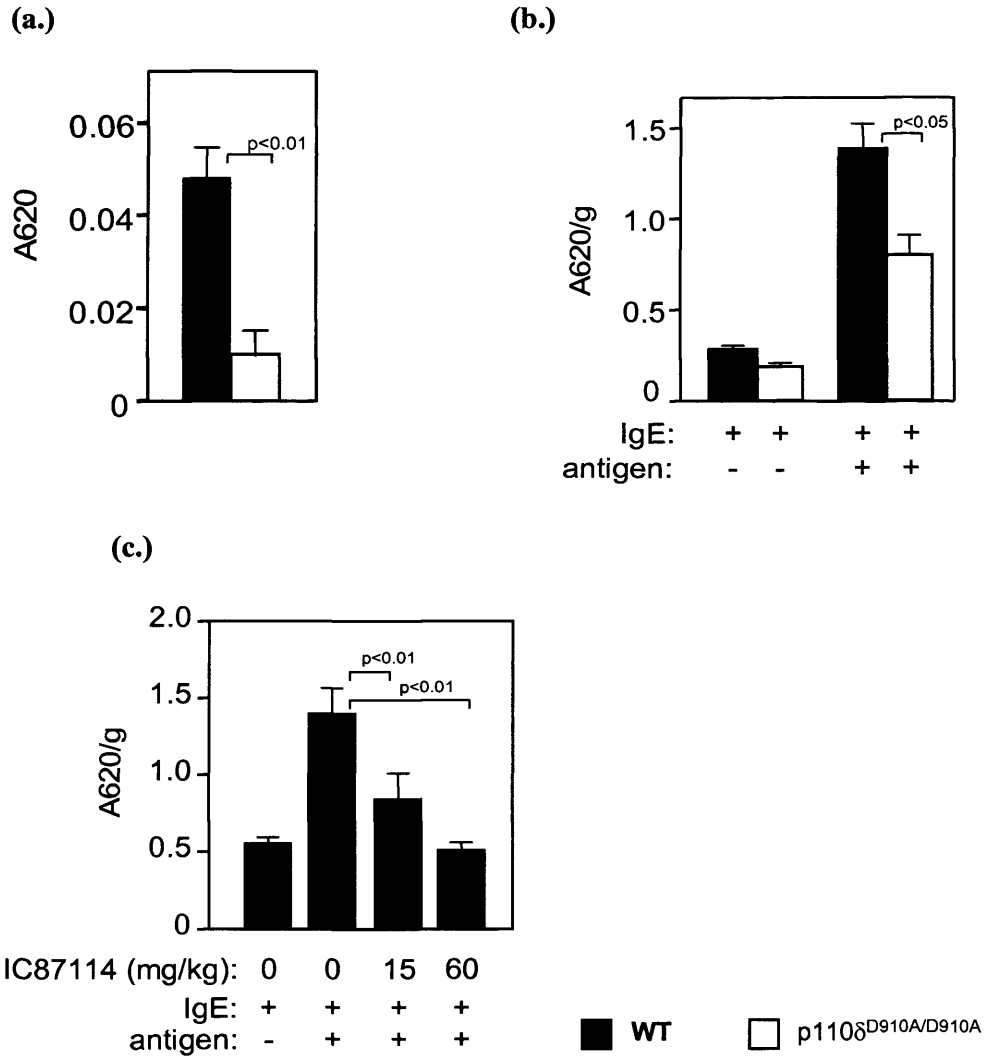


Fig 4.5 p110δ is essential for the allergic immune response (experiment carried out with help from Wayne Pearce , Ludwig Institute for Cancer Research, London, and Matthew Thomas, Novartis, Horsham, UK) ***a,*** *PCA response (ear) of WT (n=13) and* $p110\delta^{D910A/D910A}$ *mice (n=10).* ***b,*** *PCA response (back skin) of mice injected with anti-DNP IgE, challenged with saline (n=4 for WT; n=5 for* $p110\delta^{D910A/D910A}$*) or DNP-HSA (n=5 for WT; n=6 for* $p110\delta^{D910A/D910A}$*).* ***c,*** *IC87114 attenuates the PCA response (back skin) of WT mice (n=8 for each treatment group).*

4.3.2 Passive systemic anaphylaxis

Systemic exposure of antigen to a sensitized individual leads to anaphylaxis which can be life-threatening and a consequence of mast cell activation leading to vascular collapse (Wedemeyer and Galli 2000). Sensitization of mice via systemic administration of antigen-specific IgE followed 24 h later via systemic administration of antigen leads to an anaphylactic response which can be quantified by measuring vascular leakage and histamine release.

We assessed *in vivo* anaphylaxis in p110$\delta^{D910A/D910A}$ mice using histamine in the circulation as a measure of the response. However, our results have thus far been inconclusive. The main reason for this is that we observed a significantly higher *basal* level of histamine in p110$\delta^{D910A/D910A}$ mice that were sensitized but not challenged with antigen (Fig. 4.7). If we take this higher basal histamine level into account, p110$\delta^{D910A/D910A}$ mice challenged with antigen following sensitization showed substantially reduced plasma histamine levels (~60% less then WT mice) (Fig 4.7). These data and possible reasons for the elevated histamine levels will are discussed in section 4.4.4.

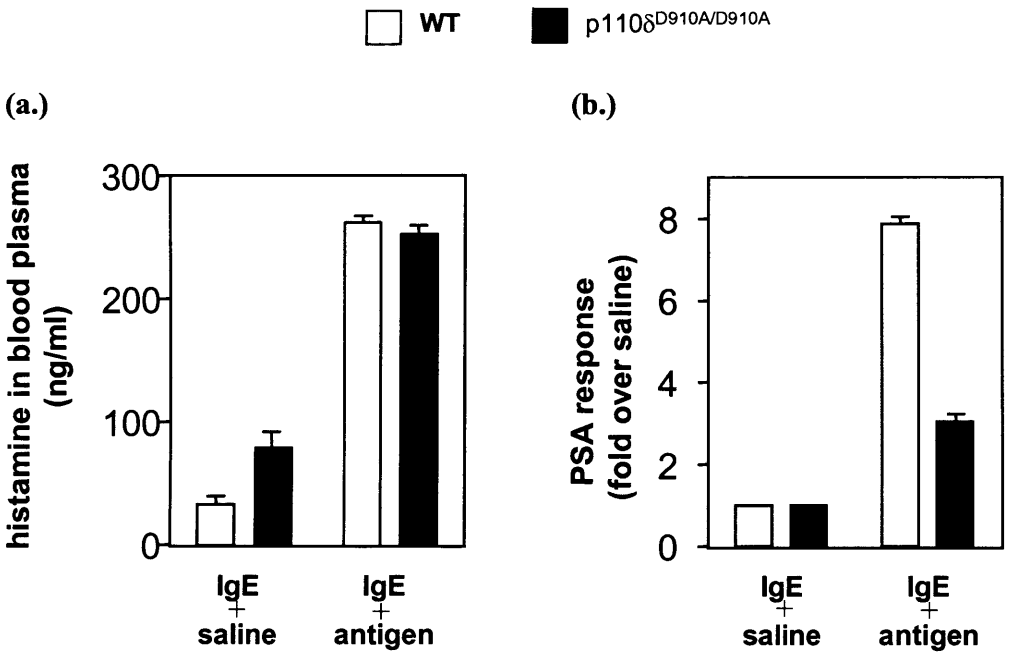


Fig 4.7 p110δ$^{D910A/D910A}$ mice have elevated basal histamine levels (experiment carried out with help from Wayne Pearce, Ludwig Institute for Cancer Research, London). *WT and p110δ^{D910AD910A} mice were sensitized with IgE (2 μg) 24 h prior to challenge with antigen (500 μg) (n = 11 for WT, n=9 for p110δ^{D910AD910A}) or saline (n =6 for WT, n=5 for p110δ^{D910AD910A}) (under terminal anaesthesia) for 2 min after which mice were sacrificed and terminal blood samples were taken for plasma histamine analysis.* ***a,*** *Sensitized but unchallenged p110δ^{D910AD910A} mice have significantly elevated plasma histamine levels.* ***b,*** *Data from (a) presented as PSA response fold over basal, this suggests that p110δ$^{D910A/D910A}$ mice are less responsive to PSA challenge.*

4.4 Discussion

Previous work has revealed that genetic deletion of Gab2 one of two major scaffolding proteins (the other being LAT) recruited to FcεRI receptor complexes upon activation, removes a major platform through which FcεRI can gain access to class IA PI(3)Ks in BMMCs. Indeed Gab2 null BMMCs have a severe reduction (up to 80%) in FcεRI stimulated PI(3)K activity, a partial reduction in calcium flux and a substantial reduction (~65 %) in degranulation (Gu, Saito et al. 2001). Gab2 null mice have impaired passive cutaneous and passive systemic anaphylaxis responses *in vivo* (Gu, Saito et al. 2001). These data led to the hypothesis that disruption of a sufficiently large quantity of class IA PI(3)K activity may mimic the defects of Gab2 deletion and block IgE-dependent anaphylaxis *in vivo*. This hypothesis is supported by data from a number of other studies using broad-spectrum PI(3)K inhibitors, the pan-class IA Δp85 inhibitor construct and p110 isoform-specific neutralising antibodies (Yano, Agatsuma et al. 1995; Ching, Hsu et al. 2001; Smith, Surviladze et al. 2001; Windmiller and Backer 2003). We found that p110δ contributes 90% of the total class IA PI(3)K activity in BMMCs (Fig 3.2c), allowing us to test the above hypothesis.

4.4.1 p110δ activity is essential for in vitro mast cell activation

The data from our studies to a large extent support the above hypothesis. Indeed, genetic or pharmacological inactivation of p110δ leads to substantial defects in IgE/antigen-stimulated PI(3)K activity (assessed using PKB phosphorylation) calcium mobilisation, cytokine release and mast cell degranulation.

Our data provide a direct correlation between PI(3)K activity and BMMC exocytosis. We also show that this PI(3)K activity not only drives the exocytosis process but also the release of pro-inflammatory cytokines such as IL-6 and TNFα the release of which is substantially diminsished upon IgE-antigen activation in the absence of p110δ activity. It is therefore possible that p110δ drives important pathways which lead to transcription and synthesis of these cytokines including NfκB and the JNK pathways and is an area which requires further investigation.

4.4.1.1 Model for p110δ participation in mast cell degranulation

Broad-spectrum PI(3)K inhibitors can attenuate up to 80% of the IgE/antigen stimulated mast cell degranulation response (Fig 4.2a). To date no study has identified a component downstream of the FcεRI cascade *critically* dependent upon PI(3)K input. Calcium mobilisation is one of the degranulation 'events' found to be particularly sensitive to PI(3)K inhibitors. Our data confirm that p110δ is important for calcium flux but does not identify a specific p110δ target which can influence calcium flux.

Overall the literature points towards PI(3)K/PIP_3 being a facilitator of translocation events, bringing together the molecular machinery (including Tec family PTK, guanine nucleotide-exchange factors and others) and thereby assisting in the formation of a 'signalosome' which promotes calcium mobilisation, PKC activation and exocytosis.

p110δ activity may also activate plasma membrane calcium channels (either directly via PIP_3 or indirectly by recruiting Tec family PTKs such BTK) which control calcium influx from the extracellular environment.

We have combined our observations from signalling and functional studies to propose a model for p110δ participation downstream of the FcεRI, as shown in Fig 4.8.

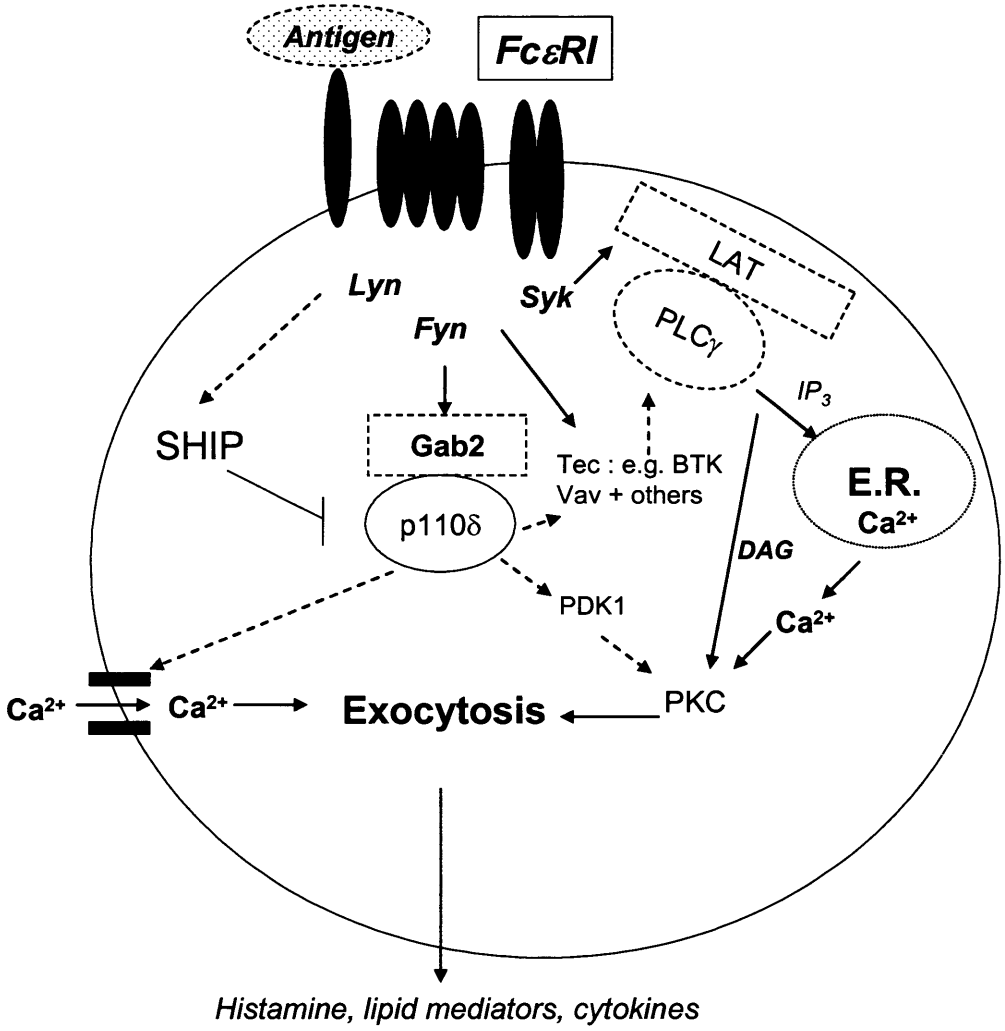


Fig 4.8 PI(3)K is a facilitator. *PI(3)K brings together different components of the calcium signalling cascade which together form a 'signalosome' centred around the adaptor protein LAT.*

4.4.1.2 p85 deletion does not disrupt PI(3)K activity

Our findings are in contrast to previously published data from p85 gene-targeted models. Partial or complete disruption of *PIK3r1* or *PIK3r2* has no effect on IgE/antigen-stimulated mast cell calcium response or exocytosis. Paradoxically

IgE/antigen-stimulated PKB phosphorylation in mast cells can be enhanced (in pan-p85α mast cells) or kinetically altered (in p85β null mast cells) suggesting that PI(3)K activity was certainly *not* reduced(Lu-Kuo, Fruman et al. 2000; Tkaczyk, Beaven et al. 2003). In other words mast cells derived from p85 KO models still have access to PI(3)K activity within FcεRI receptor complexes which is sufficient to drive PI(3)K dependent signalling pathways and mast cell exocytosis. The most likely explanation is that the remaining regulatory adaptor proteins (or up-regulation of the non-targeted isoforms) in p85 KO models can both stabilise and recruit PI(3)K compensating for the genetic deletions. This explanation suggests that class IA PI(3)K activity downstream of the FcεRI receptor has not been reduced in p85 models.

Another explanation which has been put forward is that in p85 models there may exist a pool of 'free' p110 which may be recruited independently of regulatory subunits, possibly through their C2 domain or via activated Ras, however although this is possible, regulatory subunits also stabilise p110, it is not clear how these 'free' p110 subunits would be stabilised in such an environment(Lu-Kuo, Fruman et al. 2000).

4.4.1.3 Residual PI(3)K activity downstream of FcεRI

$p110\delta^{D910A/D910A}$ BMMCs have a residual degranulation response which remains partially PI(3)K-dependent (and drives ~30% of the total degranulation event) and

can be attenuated by broad spectrum PI(3)K inhibitors. The nature of the PI(3)K(s) which provide(s) this input into FcεRI responses remains unknown.

Possible PI(3)K candidates which sustain the remaining LY294002-sensitive fraction of the degranulation response in p110$\delta^{D910A/D910A}$ and WT BMMC cells treated with IC87114 include p110α and p110β which may contribute sufficient PI(3)K activity by becoming recruited upon FcεRI ligation, despite the fact that they only appear to contribute to a minor extent to the overall class IA PI(3)K activity in BMMCs. Other candidates include the GPCR-coupled p110γ which has also been implicated downstream of IgE/antigen responses (see section 4.4.5) and the other classes (class II, III) of LY294002-sensitive PI(3)Ks (Laffargue, Calvez et al. 2002; Windmiller and Backer 2003).

4.4.2 p110δ activity is essential for human mast cell activation

Both human and murine mast cells treated with IC87114 have similar reductions in exocytosis. In a more physiological setting in which human mast cells are activated with suboptimal concentrations of antigen in the presence of SCF, exocytosis was more severely disrupted. As mast cells are maintained in the tissue environment by SCF/Kit interactions, our data suggest that within an *in vivo* context, p110δ may have a more important role in influencing mast cell activation.

4.4.3 Class I PI(3)Ks are essential for the in vivo allergic response.

Our data indicate that disruption of class I PI(3)K activity is sufficient to completely block the *in vivo* passive cutaneous anaphylaxis response. IgE/antigen activation of mast cells *in vitro* consists of two fractions, a PI(3)K-dependent fraction (80% of the

total degranulation response) and PI(3)K-independent fraction (20% of the total) which is resistant to broad-spectrum PI(3)K inhibitors (Fig 4.2a). The PI(3)K-independent part of *in vitro* degranulation is thought to be driven by PLCγ activity (Tkaczyk, Beaven et al. 2003). Our *in vivo* data indicate that any putative PI(3)K-independent PLCγ activity may not significantly contribute towards mast cell activation *in vivo*, as under conditions of 40 μM plasma dose of IC87114, there was full abrogation of the measurable PCA response (Fig 4.5). In other words, we found that all degranulation in our *in vivo* experiments appeared to be PI(3)K-dependent. Although it remains possible that at 40μM IC87114 may have off-target effects (including PLCγ) it would seem that within an *in vivo* context PI(3)K does have significant influence over PLCγ activity. The difference between *in vivo* and *in vitro* data may be explained by subtle changes signalling pathways which may be a consequence of culture adaptation which lessen the requirement for PI(3)K input into the PLCγ pathway.

4.4.4 Increased basal histamine levels in p110δ$^{D910A/D910A}$ mice

p110δ$^{D910A/D910A}$ mice passively sensitized with IgE but not challenged with antigen were found to have considerably higher levels of plasma histamine than equivalent WT mice (~58% above WT levels) (Fig 4.7). This result makes conclusions relating to systemic anaphylaxis experiments very difficult.

4.4.4.1 Increased basal histamine as a consequence of reductions in IgE- binding cells and receptors.

Increased basal histamine levels in IgE-sensitized p110δ$^{D910A/D910A}$ mice which were not challenged with antigen maybe consequence of increased numbers of circulating immune complexes within these mice. It is known that monoclonal IgE can aggregate into immune complexes which can activate cells expressing FcεRI and FcγRIII receptors (Takizawa, Adamczewski et al. 1992).

p110δ$^{D910A/D910A}$ mice are known to have a reduction in mast cells and B cells both of which express receptors able to bind IgE. In addition B cells release a soluble form of the IgE receptor FcεRII (CD23) which may be reduced in p110δ$^{D910A/D910A}$ mice as a consequence of the B cell defect (Fremeaux-Bacchi, Fischer et al. 1998; Gould, Sutton et al. 2003). It is therefore possible these reductions in interacting cells and receptors within p110δ$^{D910A/D910A}$ mice results in increased plasma IgE levels (following passive sensitization) which may form immune complexes and activate histamine releasing cells such as mast cells and basophils.

4.4.4.1 Increased sensitivity to 'cytokinergic' properties of SPE-7 monoclonal IgE antibody.

Another speculative explanation for the increased histamine levels in p110δ mutant mice might relate to increased responsiveness of these mice to 'cytokinergic' signalling of the SPE-7 monoclonal IgE utilised in our studies. Indeed, mast cell

interaction with this monoclonal IgE has been found to activate intracellular signalling cascades leading to functional responses (including survival and cytokine release), independently of antigen crosslinking (Kitaura, Song et al. 2003). However the pathway/cell which translates this feature of IgE into higher basal histamine levels in unchallenged p110$\delta^{D910A/D910A}$ mice is at present unknown.

It is also possible that the p110δ mutation results in subtle adaptation of the degranulation machinery, as occasionally increased basal (i.e. without IgE or antigen) degranulation in p110$\delta^{D910A/D910A}$ BMMCs has been observed (data not shown, ~5% above WT levels). At present, it is not clear what causes this enhanced spontaneous *in vitro* degranulation and is another area which requires additional investigation.

4.4.4.3 Loss of PI(3)K activity leads to enhanced innate immune responses.

There is genetic and pharmacological data to suggest that PI(3)K may have a negative role in the regulation of innate immune responses (Fukao, Tanabe et al. 2002; Guha and Mackman 2002; Schabbauer, Tencati et al. 2004; Aksoy, Vanden Berghe et al. 2005).

Pretreatment of dendritic cells with broad-spectrum PI(3)K inhibitors induces increased IL-12 release following stimulation with a variety of toll-like receptor ligands, including lipopolysacharide (LPS), peptidoglycan and CpG-oligodeoxy-nucleotide(Fukao, Tanabe et al. 2002). Dendritic cells derived from p85α null mice have a similar increase in IL-12 production. Furthermore macrophages treated with

broad-spectrum PI(3)K inhibitors followed by LPS stimulation have an increased production of pro-inflammatory cytokines including IL-6 and TNFα. Likewise, disruption of SHIP which is expected to result in an increase in PI(3)K activity has been reported to lead to a substantial reduction in the pro-inflammatory cytokines production in SHIP null macrophages (Guha and Mackman 2002; Fang, Pengal et al. 2004; Schabbauer, Tencati et al. 2004). These data are indicative that at the organismal level PI(3)K may have a role in maintaining the Th1/Th2 balance (Fukao, Tanabe et al. 2002).

Mast cells express a range innate immune toll like receptors and have an important role in the host defence against bacterial infections; mast cells when exposed to bacteria release a range of inflammatory mediators (including leukotrienes, histamine and TNFα) which are important in recruiting leukocytes (such as neutrophils) and help resolve bacterial infections (Echtenacher, Mannel et al. 1996; Malaviya, Ikeda et al. 1996; Mannel, Hultner et al. 1996; Puxeddu, Piliponsky et al. 2003) (Malaviya, Ikeda et al. 1996; Malaviya and Abraham 2001).

If class IA PI(3)K, as has been suggested by some of the studies detailed above, has a negative role within the innate immune system (downstream of toll-like receptors) then inactivation of p110δ may reflect a significant disruption of PI(3)K activity thereby enhancing innate immune receptor responses to bacterial infections. It is therefore possible that mast cells within $p110\delta^{D910AD910A}$ mice have an enhanced responsiveness to endogenous gut bacteria which activate the liberation of mast cell

products (including histamine) and may explain why these mice have an increased basal histamine levels.

4.4.5 Interaction between p110γ and p110δ in the allergic response?

In agreement with the essential role for p110γ downstream of GPCRs, p110γ null mice are completely resistant to increases in vascular permeability induced by adenosine (Laffargue, Calvez et al. 2002) .

p110γ is also essential for optimal IgE/antigen stimulated mast cell activation in which it participates by way of an autrocrine loop (Fig 4.9). IgE/antigen complexes activate a protein tyrosine cascade downstream of the FcεRI receptor leading to the recruitment of class IA PI(3)K activity, which is modulated by the action of the lipid phosphatase SHIP. This initial stage of mast cell activation leads to the liberation of intracellular adenosine (and other GPCR agonists) into the extracellular milieu. Adenosine acting through the A3-adenosine receptor stimulates a critical burst of p110γ-derived PIP_3 which is essential for sustained extracellular calcium influx and is sufficient to circumvent SHIP and thereby exacerbates the mast cell response leading to optimal mast cell activation.

The partial contribution of the p110γ-dependent autocrine loop towards IgE/antigen-stimulated *in vitro* BMMC degranulation is translated into a critical contribution within an *in vivo* context namely that p110γ null mice are completely resistant to IgE/antigen-dependent passive systemic anaphylaxis challenge.

These data attribute a similar function to p110γ as the one we have described for p110δ, namely an important (indirect, in case of p110γ) role downstream of the IgE/antigen-stimulated allergic cascade.

In order to resolve this issue it will necessary to carry out a parallel study on p110δ and p110γ mutant mice under similar experimental conditions. However it is possible that both of these studies have confounded by additional variables. These include the loss of kinase-independent functions (in the p110γ null mice) and by endothelial defects which may have masked the full extent of the PSA/PCA response in both strains of mutant mice, these aspects are discussed below.

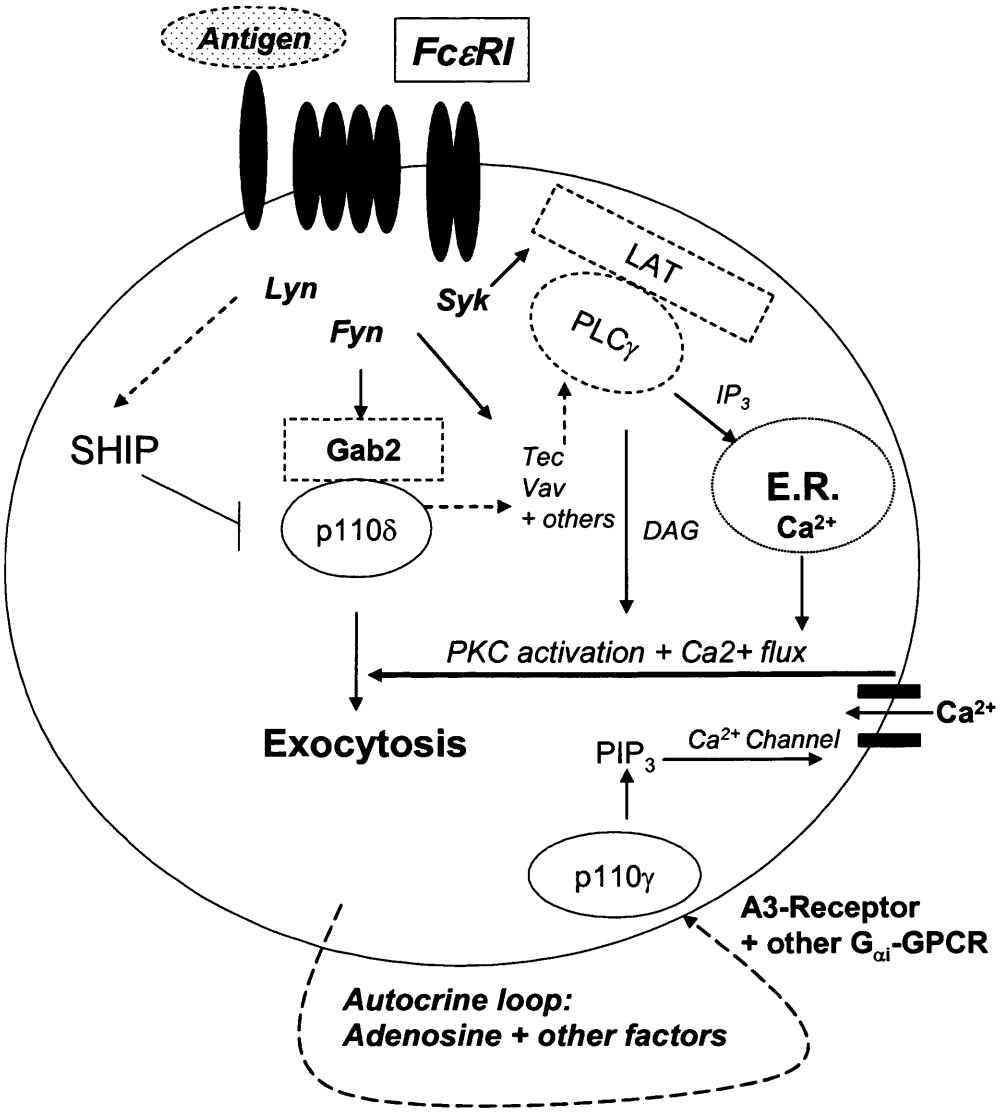


Fig 4.9 p110γ participates in the allergic response by of an autocrine loop. *Initial IgE/antigen stimulated mast cell activation leads to the liberation of autocrine GPCR agonists including adenosine which activate an autocrine loop, which provides a important burst of PI(3)K activity which overcomes SHIP down-regulation and promotes optimal mast cell degranulation.*

4.4.5.1 Disruption of a p110γ/phosphodiresterase complex inhibits cAMP metabolism.

The resistance of p110γ null mice to IgE/antigen dependent passive systemic anaphylaxis may be a result of a defect in mast cell cAMP metabolism. Deletion of p110γ may lead to a disruption of a protein complex which is important for recruiting the phosphodiesterase (PDE) enzyme to the adenosine receptor upon activation.

Cyclic adenosine monophosphate (cAMP) is a potent intracellular second messenger activated by a range of hormone receptors and GPCRs (Essayan 2001). Cyclic AMP is synthesized from adenosine triphosphate (ATP) by adenylate cyclase, and acts by binding to protein kinase A (PKA) causing dissociation of the PKA tetramer into its component monomers- two regulatory subunits with cAMP attached, and two active catalytic subunits. These events enable activation of PKA and phosphorylation of substrate proteins (Essayan 2001).

The activities of cyclic AMP are modulated by phosphodiesterases which hydrolyse the 3'-phosphodiester bond in cAMP, resulting in the formation of the corresponding inactive 5'-monophosphate (Essayan 2001). Disruption of PDE blocks hydrolysis of cAMP and causes accumulation of cyclic nucleotides, leading to increased PKA activity.

p110γ (together with other unknown molecular partners) is thought to have a scaffolding function and is important in recruiting the PDE-3B isoform to activated GPCR receptors, independently of its lipid-kinase activity (Patrucco, Notte et al. 2004). Loss of this negative control of PDE-3B upon loss-of-expression of p110γ in the p110γ KO mice is thought to lead to unrestrained PKA activity as a consequence of the failure to hydrolyse cAMP. This is thought to be the underlying cause of cardiac failure (induced by transverse aortic constriction) in mice lacking p110γ expression (Patrucco, Notte et al. 2004). The importance of this kinase-independent scaffold function was documented in a comparison of p110γ KO (lacking p110γ expression) and so-called p110γ KR mice (which express kinase-dead p110γ). Unlike p110γ KO (which have reduced PDE3B activity), p110γ KR mice (with normal PDE3B activity) are protected from cardiac tissue damage caused by increased work load. It is possible that a similar effect on cAMP is also important in mast cells.

Inflammatory cells are extremely sensitive to alterations in cAMP levels. Elevation in cAMP levels through modulation of PDE activity by inhibitors can downregulate immune cell functions including the degranulation of mast cells (Essayan 2001). Of the 11 different classes of cAMP PDE isozymes identified, both cAMP PDE3 and PDE4 are expressed in mast cells (Essayan 2001).

Extracellular adenosine is thought to play a role in inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Polosa 2002).

Adenosine is a nucleoside consisting of the purine base, adenine, in glycosidic linkage with the sugar, ribose. Extracellular adenosine is predominantly derived from the 5'-nucleotidase cleavage of adenosine 5'-monophosphate (AMP). Cellular production of adenosine is greatly enhanced under conditions of local hypoxia, as may occur in inflammation (Polosa 2002).

Mast cells are thought to play a major role in the bronchoconstrictor response to inhaled adenosine. *In vitro* adenosine can substantially enhance the release of histamine from mast cells, and is also liberated from activated mast cells following IgE/antigen crosslinking (Marquardt, Gruber et al. 1984; Marquardt and Walker 1988). Adenosine activates a number of GPCR receptors expressed on the mast cell surface including A1, $A2_a$, $A2_b$ and A3 (Forsythe and Ennis 1999; Laffargue, Calvez et al. 2002). Activation of adenosine receptors can either stimulate (A1, A2B, A3) or inhibit (A2B) histamine release (Fig 4.8).

p110γ is coupled to $G_{\alpha i}$ –type GPCRs and signals through the A3 type adenosine receptor (Laffargue, Calvez et al. 2002). Activation of $G_{\alpha i}$ type of GPCR receptors leads to recruitment of PDE and the breakdown of the cAMP messenger. It is therefore conceivable that if p110γ is important for recruiting PDE (possibly PDE3B) in mast cells, disruption of the p110γ/PDE complex will have a negative impact on mast cell activation by artificially increasing cAMP levels which would inhibit the release of mast cell derived pro-inflammatory mediators such as histamine, arachidonic acid and cytokines which may all contribute to the resistance

of p110γ KO mice to systemic anaphylaxis challenge (Fig 4.8) (Laffargue, Calvez et al. 2002).

4.4.5.2 PI(3)K expression in the endothelium

Endothelial cells have been shown to express a significant quantity of p110δ and p110γ (Puri, Doggett et al. 2004; Puri, Doggett et al. 2005). Mast cells release a broad range of pro-inflammatory mediators (histamine, TNFα, VEGF, adenosine and others) many of which can enhance vasodilation thereby increasing vascular permeability (Metcalfe, Baram et al. 1997). Some of these mediators interact with receptors known to couple to class I PI3K isoforms, and for example include VEGF and adenosine which couple to class IA PI(3)Ks (such as p110δ) and class IB, respectively. Genetic or pharmacological inactivation of one or more of these isoforms within the endothelium may also have implications for the allergic response. Both the p110δ and p110γ studies have utilised experimental protocols which rely on vascular permeability as an endpoint and a measure of mast cell activation. It is possible that normal or partially reduced mast cell activation may have been masked by vascular endothelial defects. In other words mast cell-liberated products which are able to increase vascular permeability may interact with receptors which are dependent upon class I PI(3)K activity. Thus genetic inactivation or deletion of p110δ and p110γ respectively may have reduced the sensitivity of the vascular endothelium to these agonists and may have lead to mast cell independent effects which have confounded the interpretations relating to the sensitivity of $p110\delta^{D910A/D910A}$ and p110γ null mice to PCA and PSA responses.

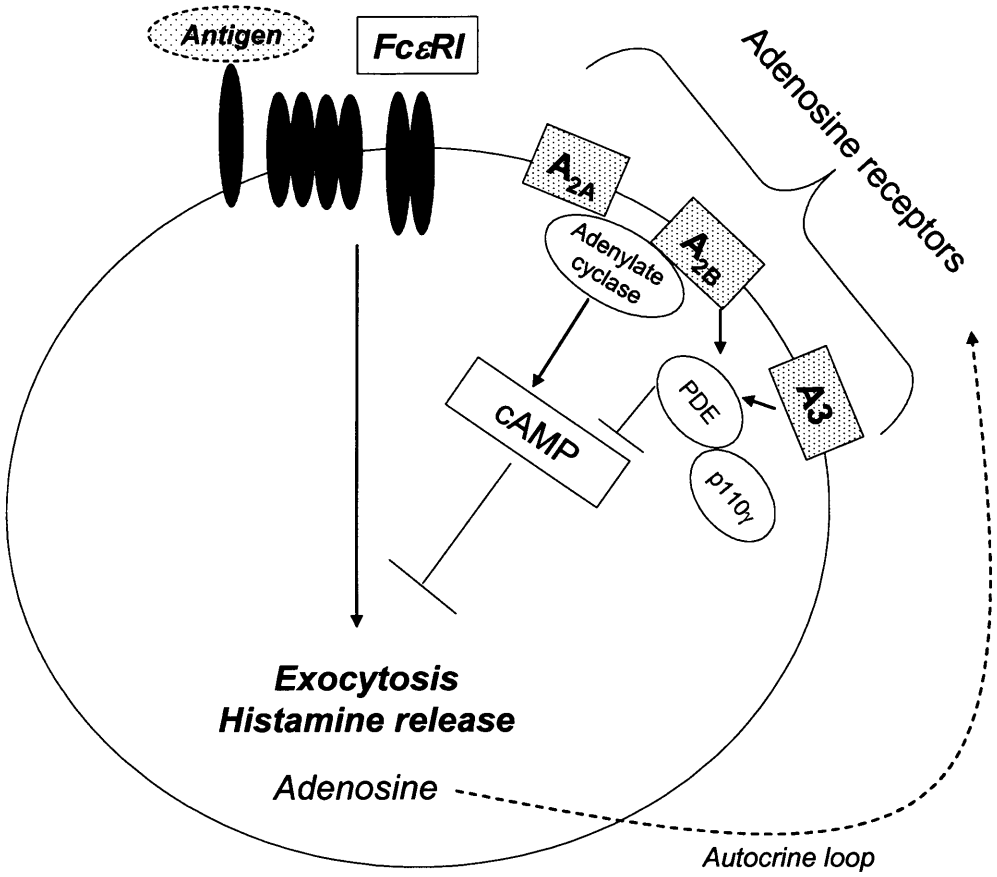


Fig 4.8 p110γ may be a scaffold for binding PDE. *Adenosine receptors can either stimulate or inhibit mast cell degranulation. p110γ couples to Gαi type of adenosine receptor (A3) which stimulates histamine release by recruiting phosphodiesterase to break down cAMP which is the product of inhibitory adenosine receptors (A_{2A}). If in mast cells p110γ is in a constitutive complex with PDE as is the case in cardiac tissue, then deletion of p110γ would impact PDE recruitment and artificially increase cAMP levels which would inhibit mast cell histamine release.*

5.0 General Discussion

Mast cells are central to IgE-dependent allergic inflammation and utilise PI(3)-*kinase* activity to support functions ranging from growth factor-induced proliferation and survival to IgE/antigen-activated exocytosis and cytokine production (Fukao, Terauchi et al. 2003). When we started our work, this evidence was mainly based on the observation that broad spectrum small molecule inhibitors of PI(3)K, such as wortmannin or LY294002, have a strong inhibitory effect on mast cell functions. While these inhibitors block all classes of PI(3)Ks, it is mainly the class IA subset of PI(3)Ks which was thought to drive a significant number of these PI(3)K-dependent responses in mast cells. Below, we give a general overview of our findings, for detailed discussions of the results, the Reader is referred to the indicated chapters.

To date, class IA-specific aspects of PI(3)K signalling have been primarily investigated using genetic deletion strategies which remove the stabilising elements of the heterodimeric p110/p85 PI(3)K complex, namely the 'p85' regulatory subunits. By removing p85 it was hoped that receptors and adaptor proteins would no longer be able to recruit / utilise the p110-kinase activity. This type of approaches provided the first genetic insight into the functions of class IA PI(3)K in mast cell biology. This work suggested that class IA PI(3)K is important for aspects of mast cell homeostasis directed through the Kit receptor but, surprisingly, appeared not to be required for mast cell activation through the FcεRI receptor (Lu-Kuo, Fruman et al. 2000; Fukao, Yamada et al. 2002; Fukao, Terauchi et al. 2003). The latter observations were surprising, given the strong inhibition of FcεRI receptor-driven

responses by broad-spectrum PI(3)K inhibitors. Of course, it was possible that class IA PI(3)Ks, contrary to the expectations, were not involved in FcεRI receptor function.

The class IA PI(3)K is a family of 3 distinct p110 catalytic isoforms which couple to one of 5 regulatory adaptor proteins. Mast cells express all 3 class IA p110 catalytic subunits including the primarily leukocyte restricted p110δ isozyme (Chantry, Vojtek et al. 1997; Vanhaesebroeck, Welham et al. 1997). Using a mouse model in which p110δ (referred to as $p110\delta^{D910A}$) has been inactivated we have assessed the role of this isoform in mast cell development (*chapter 3*), activation (*chapter 4*) and the *in vivo* allergic immune response (*chapter 4*).

Using cells derived from $p110\delta^{D910A/D910A}$ mice we have determined that p110δ is the principal source of class IA PI(3)K activity available to mast cells (*section 3.2.2*); genetic inactivation of p110δ constitutes a ~90% reduction in total class IA lipid-kinase activity; it is therefore inevitable that disruption of such a large **quantity** of PI(3)K activity will most likely have an impact on class IA PI(3)K-dependent responses and will be a be a determinant in many of the p110δ-*specific* responses described in this study. There are also some indications that there may be some **qualitative** differences between *receptor* utilisation of PI(3)K isoforms, however it is not clear if these are (1) a product of the differential recruitment of PI(3)K isoforms (possibly via scaffold proteins which associate with different PI(3)Ks); (2) intrinsic differences in p110 isoforms (i.e. differences in p110 interactions with Ras,

p110 protein kinase activity, including autophosphorylation) (3) the regulatory isoforms with which the p110s associate (p85α and p85β have additional N-terminal domains which extend their functions beyond recruiting/stabilising p110 and may recruit additional molecules which determine p110 activation status within specific receptor/adaptor complexes) (4) or a combination of the factors described above..

Impact of p110δ inactivation on growth factor-induced signalling and functional responses in mast cells

In *Chapter 3* we describe a series of phenotypes associated with p110δ inactivation and growth factor-dependent responses. We have found the IL-3 and Kit receptors have a *differential* sensitivity to genetic or pharmacological p110δ inactivation. Whereas IL-3 signalling and functional responses are almost-fully PI(3)K-dependent, they are only partially (up to ~50%) sensitive to p110δ inhibition; the residual PI(3)K activity (together with pathways which are unaffected by PI(3)K inhibition, including MAPK) can maintain *in vitro* mast cell cultures. In contrast, PI(3)K-dependent Kit-receptor mediated responses (PKB phosphorylation, proliferation, adhesion and migration) are almost completely abrogated upon p110δ inactivation. In the discussion (*section 3.3*) we highlight several intrinsic differences between the IL-3 and Kit receptors which may determine this differential sensitivity, and which can be summarised as follows. In contrast to Kit, the IL-3 receptor has no direct binding site for class IA PI(3)Ks. Stimulation of the IL-3 receptor leads to the activation of Janus-family (JAK) PTKs which phosphorylate tyrosine residues on the IL-3 receptor β-chain which allows this receptor to recruit adaptor proteins including

Gab2 and provides the receptor access to class IA PI(3)Ks. Gab2 is a Dos-family scaffold protein that contains YxxM sequences which can bind PI(3)K. As there is no known selectivity between p85/p110 and YxxM sequences it is likely that all class IA subunits can couple to Gab2 (including p110α and p110β) thus the residual PI(3)K activity downstream of IL-3 signalling may be a contribution of Gab2-associated p110α and p110β activity.

The Kit receptor can also recruit Gab2/PI(3)K to augment the SCF-induced proliferation response. Indeed, in the absence of Gab2 (Gab2 null BMMC) SCF-stimulated PKB phosphorylation and mast cell proliferation are partially reduced. It is not clear as to why, under conditions in which p110δ is inactive, the Kit/Gab2/PI(3)K does not drive at least a partial PI(3)K-dependent response. Several reasons to explain this may be put forward (1) it is possible that the Kit receptor cannot recruit sufficient Gab2/PI(3)K and therefore does not achieve a threshold to drive Kit-dependent PI(3)K signal(s)/response(s) (2) The Kit
receptor cannot recruit Gab2 as it may require an initial phase of p110δ-dependent PIP_3 to do so (Gab2 is known to have a PIP_3 interacting PH domain) (Rodrigues, Falasca et al. 2000). We have preliminary data to suggest that p110δ may be associated with the Kit receptor under basal, unstimulated conditions; however it is not clear if p110δ exclusively associates with the Kit receptor, or if any association is *non*-exclusive and that in the presence of all 3 isoforms p110δ is specifically activated over p110α and p110β (possibly by Kit receptor associated accessory molecules like Ras or Rac). (3) in the absence of a selective recruitment/association

most of the constitutively associated Kit/PI(3)K will be p110δ by virtue of a predominant expression level, this may impact on (1).

In *section 3.2.4* we have described our observation that in the presence of the p110δ-selective inhibitor IC87114, IL-3-dependent (or IL-3 co-stimulated with SCF) mast cell proliferation and expansion are severely impaired. We conclude that as the yield of viable mast cell numbers is not significantly reduced under these conditions, the observed attenuation in mast cell expansion and proliferation may be as a consequence of a *delay* in cell cycle progression. Previously published work showed that expression of a dominant-negative p85α unable to bind p110 (Δp85α) in BMMC lead to a *delay* in progression through the G1-phase of the cell cycle and a corresponding *delay* in mast cell expansion (Fox, Crew et al. 2005). We conclude that this may also be the case upon p110δ inactivation and may also explain the substantially reduced number of mast cells (up to 50% less) derived from $p110\delta^{D910A/D910A}$ bone marrow progenitors after a 4-week culture period.

Using histological analysis (*section 3.2.7 and 3.3.2*) we have determined the *in vivo* consequences of constitutive p110δ inactivation on mast cell tissue distribution; $p110\delta^{D910A/D910A}$ mice have a tissue site-selective reduction in mast cell populations (primarily within the gastrointestinal tract and the peritoneum). p110δ-activity does not appear to have a global influence over all mast cell populations; these observations are in line published data from other mouse models in which PI(3)K activity has been directly or indirectly targeted, all these data including our own fit

with the notion that class IA PI(3)K maintains the homeostasis of (a) specific subset(s) of mast cells (*section 3.3.2*).

The SCF/Kit receptor system is critical for the homeostasis of all mast cell populations as mutations which disrupt Kit receptor kinase activity severely reduce tissue all mast cell numbers. Our *in vitro* data indicate that p110δ is critical for multiple Kit-dependent responses including proliferation, migration and adhesion, therefore there appears to be a discrepancy between our *in vitro* and *in vivo* data. Several possible explanations may be put forward to account for this apparent discrepancy (1) membrane-bound SCF (which we have not tested in our *in vitro* experiments) may *not* utilise the PI(3)K pathway and is sufficient to maintain most tissue mast cell populations. In a study which compared the properties of soluble and membrane-bound SCF. It was found that both of these growth factors can recruit p85 to the activated Kit receptor and drive proliferation, membrane-bound SCF failed to activate PKB phosphorylation, which was in contrast with the strong PKB phosphorylation observed following stimulation of with soluble SCF. This study suggested that membrane-bound SCF stimulates less internalisation than soluble SCF which may be a requirement for association with downstream PI(3)K dependent signalling elements such as PKB (Trieselmann, Soboloff et al. 2003) (2) The defects in soluble SCF may be overcome in combination with other growth factors (3) Sites which appear particularly sensitive to reductions in PI(3)K activity may have (a) mast cell population(s) which has access to mainly (or only) soluble SCF and therefore is particularly sensitive to p110δ inhibition.

IgE/antigen-dependent mast cell activation and the allergic immune response

In *chapter 4* we describe our findings relating to mast cell activation and propose a model for p110δ participation downstream of activated FcεRI-complexes (*section 4.4.5*); this model is based on the evidence that $p110\delta^{D910A/D910A}$ BMMCs which have a substantial reduction in IgE/antigen-dependent secretory granule exocytosis (~50% reduction) also have a substantially attenuated calcium response. In addition we highlight that IgE/antigen-dependent degranulation of WT mouse can be substantially attenuated by pretreatment with the p110δ-selective inhibitor IC87114. That acute pharmacological p110δ inactivation mimics genetic inactivation of p110δ indicates that any potential developmental defects in $p110\delta^{D910A/D910A}$ BMMCs do not play a significant role in the diminished IgE/antigen-triggered secretory granule exocytosis phenotype. Similar observations were made in human mast cells, indicating that our findings can be related directly to the human system.

In *section 4.4.1.1* we propose two possible mechanisms by which p110δ in activated FcεRI complexes can influence the calcium response: (1) by translocating/activating molecules into/within the LAT calcium 'signalosome' and thereby augmenting the activity of PLCγ (known PI(3)K sensitive molecules involved in calcium mobilisation which may be effected by this PI(3)K activity include Tec family PTK (such as BTK) and GEFs) (2) direct / indirect (via BTK) activation of plasma membrane calcium channels to allow influx of calcium from the extracellular environment.

However our model is in conflict with the broadly accepted idea that class IA PI(3)K is *not* significantly involved in the calcium-mobilising steps downstream of the activated FcεRI receptor (Blank and Rivera 2004). Recently it has been shown that BMMC degranulation can be dissected into two events (1) the calcium-independent microtubule-dependent translocation of granules to the plasma membrane and (2) calcium-dependent membrane fusion and exocytosis (Nishida, Yamasaki et al. 2005). Class IA PI(3)K is thought to participate in the events described under (1) which it can influence through numerous PI(3)K-sensitive effector proteins via its interaction with an regulation of smalll GTPases via GEFs and GAPs, many of which are known to be involved in cytoskeletal reorganisation which can contribute to granule translocation events. However the critical contribution of class IA PI(3)K in the calcium-independent steps is thought by some to be through its activation of the calcium-*in*dependent δ isoform of PKC (discussed below).

The activated FcεRI-receptor most likely accesses class IA PI(3)K indirectly via the scaffold protein Gab2. Indeed, genetic deletion of Gab2 or the upstream Src family PTK Fyn, essential for Gab2 phosphorylation, leads to a severe attenuation of FcεRI-dependent PI(3)K activity (90% and 80% in Fyn- and Gab2-null BMMCs, respectively, assessed using both PIP_3 analysis and PKB phosphorylation), and BMMC secretory granule exocytosis (reduction of ~80% and ~65% in Fyn and Gab2 null BMMCs, respectively), in the *absence* of a significant defect in the calcium response. As a consequence of this reduction in PI(3)K activity, Fyn- and

Gab2-deficient mice are resistant to IgE/antigen-dependent PSA (measured by histamine release) (Gu, Saito et al. 2001; Parravicini, Gadina et al. 2002).

The *calcium-independent* PKCδ has been put forward as the critical downstream target of the Fyn/Gab2/PI(3)K pathway, essential for complimenting the calcium-dependent exocytosis process (downstream of Lyn/Syk/LAT). In Fyn-deficient BMMCs PKCδ phosphorylation is substantially diminished and is thought to be the reason why these cells have a severe attenuation of degranulation (Parravicini, Gadina et al. 2002), upon this evidence and the lack of a significant reduction in the calcium response it was proposed that PKC dependent secretory granule exocytosis is essential for FcεRI-dependent mast cell degranulation and that Gab2/PI(3)K is critical for PKC activation. The model that was proposed can be summarised as:

FcεRI => Fyn > Gab2 > PI(3)K > PDK-1 >PKC = Secretory granule exocytosis

A possible explanation which can accommodate both our own data and the model described above is if the p110δ^{D910A} mutation disrupts both the Gab2 and LAT associated PI(3)K activity. Genetic deletion of Fyn or Gab2 removes the pool of class IA PI(3)K which activates PKC which is separate from the pool of PI(3)K which associates with LAT. Recently it has been shown that SLP-76(Shim, Moon et al. 2004) (which associates with LAT via the Gads adaptor protein) can directly bind the p85 subunit of PI(3)K. Deletion of SLP-76 attenuates calcium responses and the allergic response. Thus it is possible that the p110δ^{D910A} mutation disrupts both the

Gab2 and the LAT associated class IA PI(3)K. In other words the p110δ^{D910A} mutation provides a more complete understanding of PI(3)K participation in FcεRI-dependent responses.

Important areas for future work will include analysis of the PI(3)K which associates with Gab2 and that which associates with LAT (Wilson, Pfeiffer et al. 2002). As the principal class IA PI(3)K p110δ mutation would also be expected to impact the Gab2 pathway and PKC activation. It will therefore be of interest to see how PKC activity is affected upon p110δ inactivation.

p110δ inactivation can attenuate the in vivo allergic response

In *section 4.4.3* we discuss our findings relating to the role of p110δ in the PCA and PSA response. We have found that genetic or acute pharmacological inactivation of p110δ can substantially attenuate the PCA response to a level which is consistent with our *in vitro* mast cell activation data (~40-50% reduction). Furthermore at higher doses of IC87114 (where it may possibly start to affect the activity of p110γ), the PCA response can be completely blocked. The role of p110δ in the IgE/antigen-dependent PSA response is less clear. A confounding factor in these studies is the observation that p110δ$^{D910A/D910A}$ mice passively sensitised with IgE but not challenged with antigen already have a higher basal level of histamine. Several possible reasons which may explain these data have been put forward in *section 4.4.4.*

Interactions between p110δ and p110γ

In *section 4.4.5* we discuss what has been referred to recently as a paradox between p110δ and p110γ (Wymann and Marone 2005). We put forward the suggestion that genetic deletion of p110γ may have affected a p110γ/PDE complex which is important for cAMP metabolism downstream of the autocrine loop. We also discuss the possibility that a defect in the endothelium could have confounded the interpretation of *in vivo* experiments in our own study and the p110γ study, both of which have used vascular permeability as a measure of mast cell activation.

Incomplete disruption of PI(3)-kinase activity and p85 specific functions in p85-'KO' models

Some of our observations are in conflict with data obtained from p85 KO studies. p85 KO strategies applied to date have removed only a fraction of the regulatory PI(3)K subunits available to mast cells, and it is not unlikely that the remaining regulatory subunits provide at least some of the PI(3)K activity downstream of activated receptors. This 'reduced' PI(3)K input may be insufficient in some cases to achieve the necessary thresholds to drive a functional response and lead to the reported phenotypes in these mice. However one data obtained from p85α-only null mice (in which p55α and p50α remain intact) provides data implying that some of the reported phenotypes may be a unique feature of disrupting the *full-length* p85α subunit. BMMCs derived from these mice express normal levels of p110δ with some reduction in p110α expression (Fukao, Yamada et al. 2002). As we have shown

p110δ is the primary isoform downstream of the Kit receptor, thus it is not surprising that SCF stimulation of p85α null BMMCs leads to only a minor reduction in Akt/PKB phosphorylation (~30% reduction); under these conditions p110δ coupled to the non-targeted p55α, p50α (which are both up-regulated in p85α-null mast cells)and p85β adaptor proteins can bind to the activated Kit receptor providing a signal which maintains most of the Kit/PI(3)K activity (~70% PKB signal). However, despite the fact that under these conditions PI(3)K activity appears only minimally reduced, the impact on Kit-driven proliferation appears to be severe, indicating that disruption of full length p85α in the absence of a significant defect in PI(3)K recruitment/activity is sufficient to impair Kit responses. In other words, full length p85α appears to have a rather specific role downstream of the Kit receptor. This may relate to the additional N-terminal domains present in p85α (and p85β) which extend their functions beyond p110 stabilisation. These include a BH domain which can interact with Rac and SH3 domains which can interact with proline-rich regions. Deletion of full length p85α appears to have a substantial impact on the JNK (60% reduction) pathway which is downstream of Rac activation (Fukao, Yamada et al. 2002). Thus it would appear that although the remaining regulatory adaptors can couple to Kit, in the absence of full length p85α they cannot compensate adequately for p85α-specific responses. We also find that JNK phosphorylation is partially disrupted in p110δ KI BMCCs (approx 60%, data not shown). This would indicated that p85α-dependent responses are also to an extent PI(3)-*kinase* dependent.

Kit / p85α-specific coupling does not appear to be relevant to other PI(3)K-dependent receptors which unlike the Kit receptor (which can bind PI(3)K directly) recruit PI(3)K indirectly via scaffold proteins such as Gab2. p85α null BMMCs (both p85α-only KO and pan-p85α KO) have no defects in IL-3-induced proliferation or IgE/antigen-stimulated degranulation. Gab2 null BMMCs stimulated with IL-3 or IgE/antigen complexes have a substantial reduction in PKB phosphorylation (~75% and 80% respectively) (Xie, Ambudkar et al. 2002). Although it is not clear how the residual PI(3)K couples to these receptors in the absence of Gab2, it is clear that this scaffold is the primary access to class IA PI(3)K activity downstream of IL-3 and FcεRI receptors. Like most receptor and adaptor proteins which have YxxM sequences, Gab2 is not expected to have any preference for binding specific regulatory adaptor subunits (Vanhaesebroeck, Leevers et al. 2001; Liu and Rohrschneider 2002). Thus it seems likely that in the absence of *PIK3r1* gene products, the remaining regulatory subunits coupled to p110 are recruited to Gab2 and provide necessary PI(3)K input to drive both IL-3 and FcεRI responses. Our data indicate that p110δ is the principal class IA PI(3)K in mast cells therefore it would seem likely that most of the regulatory subunits are in complex with p110δ, thus *most* of the Gab2 recruited PI(3)K in $p110\delta^{D910A/D910A}$ BMMCs will be 'kinase-dead' and thus our data to a *large* extent are compatible with findings from the Gab2 study (Gu, Saito et al. 2001).

The importance of careful gene-targeting

Mouse gene targeting is a powerful tool for dissecting signalling pathways. However, like all molecular or pharmacological tools there is the potential for indirect or off-target effects. Genetic deletion strategies are prone to 'artificial phenomena' which may be consequence of losing important protein-protein interactions and in the case of molecules which are part of a large family, deletion of a specific member(s) may leave room for promiscuous signalling by the non-targeted isoforms. Gene inactivation approaches are also prone to 'knock-on' effects such as the creation of a dominant-negative acting proteins or differentiation phenotypes as a result of ablation of early constitutive gene function, which can lead to compensatory mechanisms.

However it is possible to circumvent or at least reduce such scenarios by careful gene targeting. A dominant-negative is by definition is any mutation whose gene-product adversely affects the normal, WT gene-product within the same cell or organism. An example of this scenario in the case of class IA PI(3)Ks would be the creation of a regulatory subunit mutation such that adaptor proteins can no longer interact with p110 subunits (such as the Δp85α). This type of mutation would have a global effect on all class IA PI(3)K signalling. In contrast, the $p110\delta^{D910A}$ mutation specifically interferes with the p110δ molecule such that it is expressed as a constitutively inactive but structurally intact protein. $p110\delta^{D910A}$ is able to fulfil any kinase-independent functions (including scaffolding) and to take its 'normal' molecular place within signalling complexes downstream of receptor activation.

This strategy is not expected to have any effect on the functions of p110α or p110β which remain active and free to take their 'normal' place within receptor complexes; therefore it is *unlikely* that the $p110\delta^{D910A}$ creates a dominant-negative system.

The most relevant problem for any genetic approach involving the constitutive deletion or inactivation of a molecule(s) is the potential for developmental defects. We cannot exclude that developmental defects may contribute to the severity of some of the reported phenotypes; however we have also utilised a pharmacological p110δ selective inhibitor and report that acute inactivation mimics our genetic data, strengthening our conclusions on the importance of p110δ in mast cell biology. Overall the best strategy for dissecting signalling pathways is to use a combination of conditional 'knock-out' and 'knock-in' strategies combined with pharmacological and molecular approaches.

Summary

In summary our data provide new insight into the role of p110δ within a mast cell context and identifies important new functions for this PI(3)K in aspects of mast cell homeostasis, activation and the allergic immune response. Our data indicate that p110δ inhibitors could be of clinical relevance in the treatment of mast cell pathologies including allergic disease. Future p110δ inhibitors will most likely have a potent immune-suppressive effect by directly targeting the mast cell and by indirectly targeting immune cells which have a profound influence over mast cell biology, including the B-cell which has previously been shown to have a critical need for p110δ activity.

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